PHD

Synthesis and Biological Studies on ‘Smart’ Iron Chelator Molecules

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CHAPTER 4: RESULTS AND DISCUSSION (PART III)

Aminocinnamoyl-caged iron chelators.

4.1. Synthesis of aminocinnamates

The 2-aminocinnamoyl class of PRPGs are closely related to the hydroxycinnamoyl caging group described in Chapter 3, and their photochemical properties were also initially investigated by Porter et al.\cite{108} It has been shown that aminocinnamates such as 139 (Scheme 4.1) can undergo photolytic cleavage to release two photoproducts, one of which is a cyclic carbostyril or 3-methyl-quinolin-2-one such as 140a.\cite{168}

![Scheme 4.1. One of the first reported examples of an aminocinnamate which is photolytically cleaved to yield a carbostyril cyclic photoproduct (140a).](image)

One observation made by Porter et al. is that aminocinnamates generally absorb at longer wavelengths compared to their corresponding hydroxycinnamate analogues. It has also been found that mono alkyl substitution on the aniline nitrogen results in red-shifted absorption maxima, whereas di-alkyl substitution results in blue-shifted absorption. As with the hydroxycinnamates, various substitution patterns on the aromatic ring result in changes to the $\lambda_{max}$ of aminocinnamates which could be useful to “fine-tune” photorelease at specific wavelengths. For this reason, the photochemical behaviour of iron chelators caged with the 2-aminocinnamoyl group merited investigation, as they could provide a viable alternative to the 2-hydroxycinnamoyl caging group because of their potential to absorb at longer wavelengths. Although some carbostyril derivatives have been reported to have antioxidant properties,\cite{169} to our knowledge there have been no studies which describe their potential as photoprotectants; either as UV filters or by virtue of any apparent antioxidant effects.
In a similar way to hydroxycinnamoyl CIC preparation, it was envisioned that aminocinnamate CICs should be accessible via the corresponding cinnamic acid scaffold; however the prerequisite cinnamic acids are not commercially available. We therefore adapted a method reported by Porter et al.\cite{108} Starting from commercially available 2-nitrobenzaldehydes (142a-e) the corresponding olefinic ethyl ester (143a-e) were obtained in excellent yield (78-99\%) by Wittig reaction, with only the (E) stereoisomer isolated (Scheme 4.2). Stereochemistry of 143a was confirmed by X-ray crystallography (see appendix). Hydrolysis of the esters with aqueous NaOH in a mixture of THF/EtOH resulted in complete saponification, and subsequent acidic workup gave high recovery of carboxylic acids 144a-e in over 93\% yield. Previous work in our laboratory had attempted chemoselective reduction of the aromatic nitro group to the amine with SnCl2, however this had resulted in the copious generation of side-products by TLC analysis. Although reduction was achieved by heating cinnamic acids 144a and 144b in an aqueous mixture of iron and iron sulfate at reflux; yields were consistently very poor, and isolation difficult.\cite{170} Variations of this method included substitution of ferrous sulfate with ammonium chloride, or the use of sodium sulfide as a reducing agent; however no reaction appeared to occur with these conditions. Eventually, chemoselective reduction was successfully accomplished with Fe powder and AcOH in aqueous EtOH as described by Jung and Park\cite{171} which gave the expected corresponding anilines 145a-d in good or excellent yield; however for dimethylamino derivative 145e, these conditions generated extensive side-products by TLC analysis, from which the expected product could not be isolated by normal workup conditions or by column chromatography.

<table>
<thead>
<tr>
<th>R</th>
<th>4,5-(OMe)2</th>
<th>4,5,-methylenedioxy</th>
<th>H</th>
<th>4-CF3</th>
<th>4-NMe2</th>
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<tr>
<td>142-45a</td>
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<td>142-45b</td>
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<td>142-45e</td>
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Scheme 4.2.
Preparation of 2-aminocinnamic acids 145a-d
Reagents and conditions:
a. ECETP, toluene, 65 °C, overnight, 78-99\%;
b. 1 M NaOH, THF/EtOH (4:1), 40 °C,
onight, 93-99\%;
c. Fe, AcOH, 66% EtOH (aq.), 1 h, 60-99\%
With the required aminocinnamic acids in hand, coupling with various ICs to give the corresponding aroylhydrazone esters could be attempted using EDC 146 as a coupling agent to activate the carboxylic acid, and a catalytic amount of DMAP (147, 0.1 mmol) as a nucleophilic acylation catalyst. When these conditions were applied with methylenedioxy derivative 145b and SIH, the expected product was obtained but only in 26% yield. It was postulated that this might be due to either incomplete carboxylic acid activation, or intramolecular rearrangement of the O-acylisourea intermediate (Scheme 4.3). To avoid this, it was decided to add a stoichiometric amount of DMAP, so that the O-acylisourea would be completely converted to an acylpyridinium species before rearrangement could occur. This gave a significant improvement as seen with the 4,5-dimethoxy analogue 148a, which was obtained in 73% yield (Scheme 4.4).

Scheme 4.3. Possible intramolecular rearrangement of the acylisourea intermediate to give the unwanted amide (left branch) or highly electrophilic acyl-pyridinium species (right branch), for subsequent reaction with aroylhydrazone iron chelators.
Although isolation of the pure CIC could be achieved by column chromatography, this process was complicated by the fact that SIH and the desired CICs shared almost identical Rf values by TLC analysis. Separation of uncoupled SIH and the desired aminocinnamoyl-CIC by column chromatography therefore was a painstakingly slow process, and ways in which SIH coupling could be maximised to avoid this issue were therefore considered. Although the acylpyridinium species should be highly susceptible to nucleophilic attack from the phenolic oxygen of SIH, it was thought that the phenoxide anion of SIH would attack even more readily. To accomplish this, a solution of SIH was first treated with an organic base, diisopropylethylamine (DIPEA), before being added to the activated cinnamic acid mixture. It was found that all SIH was consumed under these conditions, allowing for facile isolation of CICs 148a-b in 79% or 71% yield respectively (Scheme 4.4). These conditions were also used to prepare aminocinnamoyl-PIH derivatives 149a-d (42-82%), which underwent subsequent desilylation as described in Chapter 3 to give the desired CICs 150a-d in 50-63% yield.

Scheme 4.4. Coupling of aryl substituted cinnamic acids 145a-d to either 10 or silyl-protected PIH 117, and desilylation of the latter. Reactions and conditions: a. (i) EDC, DMAP, DMF, 0 °C, 5-10 min, (ii) 10, DIPEA, RT, overnight, 71-79%; b. EDC, DMAP, DMF, 0 °C, 5-10 min, (ii) 117, DIPEA, RT, overnight, 42-82%; c. TBAF, AcOH, THF, RT, overnight, 50-63%.
4.1. Synthesis of 3-methyl-quinolin-2-ones

The release of a cyclic, coumarin-like quinolin-2-one fragment or carbostyril following the photolysis of aminocinnamates has already been described,\textsuperscript{[101]} and thus it is useful to obtain these compounds as references for uncaging experiments, and to evaluate their biological activity in relevant cell lines, for example any photoprotective effects. Unfortunately the carbostyrils are not readily available from commercial sources, and an investigation into the synthesis of these compounds was therefore necessary.

We first tried to prepare the 4,5-dimethoxy and 4,5-methylenedioxy carbostyrils 140a-b by a method reported by Jung and Park,\textsuperscript{[171]} from whose work the chemoselective nitro reduction described earlier had been adapted. Starting from the commercially available 2-nitrobenzaldehyde, reduction to the corresponding aromatic amines 151a-b was followed by N-alkylation, which furnished 152a-b in excellent yield (Scheme 4.5). According to the literature, it should then have been possible to access the desired quinolin-2-ones by base-catalysed cyclisation, with caesium carbonate being the choice of base employed by the authors; however in our hands no reaction was observed with these conditions. No change was observed when an organic base in the form of DBU was used instead, or when enolisation with sodium hydride was attempted.

\begin{scheme}
\begin{align*}
\text{142a-b} & \xrightarrow{a} \text{151a-b} \xrightarrow{b} \text{152a-b} \xrightarrow{c, d, e} \text{140a-b}
\end{align*}
\end{scheme}

\textbf{Scheme 4.5.} Initial attempt to prepare carbostyrils 140a-b by base-catalysed cyclisation of N-propanamides 152a-b.

\textbf{Reagents and conditions:}
\begin{itemize}
  \item a. Fe, AcOH, EtOH/H\textsubscript{2}O (2:1), reflux, 5-20 min, 89-95\%.
  \item b. Propionyl chloride, pyridine, toluene, RT, 20 min, 82-96\%.
  \item c. Cs\textsubscript{2}CO\textsubscript{3}, DMF, 60 °C, overnight, no reaction.
  \item d. DBU, DMF, 60 °C, overnight, no reaction.
  \item e. NaH, DMF, 60 °C, overnight, no reaction.
\end{itemize}

\begin{table}
\begin{tabular}{|c|c|}
\hline
R & 140a, 142a, 151-2a \ 4,5-(OMe)\textsubscript{2} \\
140b, 142b, 151-2b & 4,5-methylenedioxy \\
\hline
\end{tabular}
\end{table}
As a result, a second synthetic route was explored, based on the preparation of quinolin-2-ones as reported by Chen and coworkers whereby the desired product can be obtained via a palladium-catalysed intramolecular cyclisation.\cite{172} To achieve this, O-silylated aniline derivative 154 (Scheme 4.6) was N-alkylated and subsequently deprotected under basic conditions to furnish the $\alpha,\beta$-unsaturated amide 155 in 66% yield over 2 steps.

Transformation to the corresponding benzyl bromide derivative however as described by Chen et al. was problematic as treatment of 155 with phosphorus tribromide gave multiple products, with the expected bromide (156a) isolated in only 21% yield. When alternative brominating agents such as N-bromosuccinimide or carbon tetrabromide were employed, no indication of product formation was observed. We also found that 156a readily underwent degradation after storage in chloroform, even if stored at 2-8 °C in solution for 24 h. To circumvent these issues, the benzyl chloride was considered as an alternative, as it should exhibit a higher level of resistance to nucleophilic attack making it less susceptible to degradation. Furthermore, Chen et al. reported that the chloride derivative also undergoes palladium-catalysed cyclisation, albeit in a lower yield. Chlorination of acrylamide 155 was accomplished to give 156b in good yield, with no degradation observed after standing in chloroform at RT for 72 h.

![Scheme 4.6. Preparation of halogenated acrylamide precursor 157](image)

Reagents and conditions:

a. TMSCl, Et$_3$N, THF, RT, overnight, 87%

b. (i) H$_2$C=CHCOCl, Et$_3$N, DCM, 0 °C, 30 min,
   (ii) K$_2$CO$_3$, MeOH, RT, 90 min, 66%

c. PBr$_3$, DCM, 0 °C, 30 min, 21%

d. (i) SOCl$_2$, DCM, -10 °C, 30 min, (ii) RT, 4 h, 67%
e. AcCl, CaH$_2$, THF, RT, 72 h, 51%
According to the literature, the authors claimed that acetylation at nitrogen to give the tertiary amide was necessary to improve substrate reactivity in the cyclisation step. N-acetylation with acetyl chloride and CaH$_2$ as described by Chen et al. furnished 157 in a yield of 25%, the poor yield being a result of incomplete starting material consumption. It was found that a reaction time of 72 h was required to achieve a sufficient level of reaction progression, whereby the yield increased to 51%, although no further improvement was observed with the addition of DMAP or by increasing the stoichiometric ratios of base.

Reaction of acrylamide 157 under Heck-type conditions using Pd$_2$(dba)$_3$ and the ligand DPPF gave three distinct spots by TLC analysis, which is likely to correspond to the mixture of cyclic products that are described in the literature (158a-c and 140c); however a considerable amount of starting material was also observed on TLC. In this instance, these compounds were not isolated, but addition of DBU and heating at reflux for a further 3 h resulted in one major product by TLC analysis which was isolated as the desired 3-methyl-quinolin-2-one 140c which was thus obtained although only in a modest yield of 14% over 2 steps (Scheme 4.7).

Scheme 4.7.
Palladium-catalysed cyclisation of acrylamide 157 to give the 3-methylquinolin-2-one compound 140c.
Reagents and conditions: a.(i) Pd$_2$(dba)$_3$, DPPF, Et$_3$N, MeCN, reflux, 5 h, (ii) DBU, reflux, 3h, 14%.
With the carbostyril 140c now in hand, preparation of the 4,5-dimethoxy analogue was attempted using the same synthetic route described above. Since the corresponding 2-aminobenzyl alcohol was not commercially available, nitroveratraldehyde (142a) was instead chemoselectively reduced to its aniline counterpart 151a as described earlier, and subsequently alkylated at nitrogen to afford the acrylamide 159 in 45% yield (Scheme 4.8). Reduction of the aldehyde to the benzyl alcohol was achieved under mild conditions using NaBH₄ which gave 160 in 91% yield; however the chlorination conditions which had been used previously were unsuccessful and 161 was only obtained in very poor yield (<4%). Transformation of benzyl alcohol 160 into multiple products was observed by TLC, and the possibility that 161 was susceptible to acid-induced degradation during chromatography on silica gel was disproven when treatment of the column with pyridine to neutralise the gel gave no improvement.

Scheme 4.8. Attempted preparation of chloroacrylamide derivative 161 for subsequent cyclisation to the corresponding carbostyril. Reagents and conditions: a. Fe, AcOH, EtOH/H₂O (2:1), reflux, 5 min then RT 15 min, 89%; b. H₂C=CHCOCl, Et₃N, DCM, 0 °C, 25 min, 45%; c. (i) NaBH₄, iPrOH, RT, 30 min, 91%; d. SOCl₂, DCM, -10 °C → RT, 4 h, 4%.

As the synthetic route described in Scheme 4.8 was not suitable for the dimethoxy substituted compound 161, an alternative strategy was considered. Meth-Cohn et al. have described the synthesis of quinolin-2-ones via 2-chloroquinoline-3-carbaldehydes, which in turn can be prepared by Vilsmeier-Haack formylation of the corresponding N-arylacetamides 163a-b (Scheme 4.9).[173] Subsequent formylation under Vilsmeier-
Haack conditions as described in the literature proceeded to give the desired 2-chloro-3-formylquinolines (164a-b) which were obtained in good yield following purification by recrystallisation or column chromatography (40% or 61% respectively). Hydrolysis of the 2-chloroquinolines with hot aqueous AcOH furnished the expected 3-formylquinolin-2-one derivatives 165a-b in good or excellent yield (61% or 83% respectively).[^174] Despite concerns regarding the stability of the methylenedioxy moiety, which is known to undergo acid-induced cleavage, there was no indication by TLC analysis that hydrolysis of the methylenedioxy ring had occurred. Finally, acid-facilitated reduction of the aldehyde on the 3' position gave the desired 3-methyl-2-quinolin-2-ones 140a-b in reasonable yield (48% or 59% respectively).[^175]

**Scheme 4.9.** Successful Preparation of 3-methyl-2-quinolin-2-ones 140a-b via Vilsmeier formylation. *Reagents and conditions:* a. MeCOCl, Et₃N, DCM, 15-20 min, 70-75%; b. (i) DMF, POCl₃, 85 °C, 4 h, (ii) H₂O, RT, 10 min, 40-61%; c. AcOH/H₂O (2:1), reflux, 8 h or overnight, 61-83%; d. Et₃SiH, TFA, RT, overnight, 48-59%.
4.3. Decaging experiments

4.3.1. UV absorption spectra

The absorption spectra of aminocinnamoyl-PIH compounds 150a-d is shown in Figure 4.1. Note how all derivatives exhibit an absorption maximum between 280-300 nm, with the dimethoxy and methylenedioxy derivatives showing the most red-shifted absorption profiles. The trifluoromethyl derivative 150d interestingly shows a unique absorption maximum around 240 nm.

![UV absorption spectra of aminocinnamoyl-caged PIH derivatives 150a-d in EtOH at a concentration of 40 μM.](image)

**Figure 4.1.** UV absorption spectra of aminocinnamoyl-caged PIH derivatives 150a-d in EtOH at a concentration of 40 μM.
Figure 4.2 compares the UV absorption profiles of 4,5-dimethoxyaminocinnamoyl-caged SIH (148a) and its corresponding PIH derivative (150a), where it is evident that both compounds exhibit a similar level of absorption within the UVA region.

Figure 4.2. UV absorption spectra of 4,5-dimethoxyaminocinnamoyl-caged CICs 148a and 150a in EtOH at a concentration of 40 μM.
4.3.2. Stability of aminocinnamoyl-caged aroylhydrazones to visible light

The CICs 148a-b and 150a-c appear to undergo a minor degree of photolysis when exposed to ambient light for a period of 16 h at RT (Figure 4.3, A-E); however the 4-trifluoromethyl derivative 150d appears to undergo a considerably more extensive level of uncaging (F) under the same conditions.

Figure 4.3. HPLC chromatograms of aminocinnamoyl-CICs following exposure to ambient light for 16 h.

Considering the UV absorption profile of the aminocinnamoyl-CICs, which suggests that only minimal absorption occurs above 400 nm, it is surprising that these compounds exhibit this behaviour following exposure to visible light.

It has been reported recently that fluorescent light bulbs can emit 'significant' levels of UVA and UVC radiation where UV-absorbing phosphor coatings on the bulb have been compromised.[176] An alternative explanation is that the CICs are undergoing photolysis from exposure to small levels of UV radiation emitted by the fluorescent light bulbs used in this experiment.
4.3.3. Single dose UVA-decaging

Decaging profiles of the aminocinnamoyl-caged SIH derivatives **148a-b** by HPLC analysis are shown below in Figures 4.4 and 4.5 respectively. At a dose of 250 kJ/m², photolysis appears to be complete and occurs in a clean fashion, with two new photoproducts observed for both CICs. The retention times for these signals correlate with those seen for the expected reference compounds (chromatograms C and D). The retention time however of the two photoproducts, namely SIH and the corresponding 3-methyl-quinolin-2-one are very similar (Figures 4.4 and 4.5, B(i)) and a higher resolution is necessary to clearly elucidate these signals (Figures 4.4 and 4.5, B(ii)).

![HPLC chromatograms](image)

**Figure 4.4.** HPLC chromatograms of 4,5-dimethoxyaminocinnamoyl-SIH derivative **148a**, showing the intact CIC (A) and the UVA (250 kJ/m²) irradiated compound (B (i)), which is magnified in B (ii) for higher resolution. The 'naked' SIH molecule **10** (C) and corresponding quinolin-2-one photoproduct **140a** (D) are shown for reference, along with a co-injection of the irradiated CIC and SIH (magnified, E).
Unlike its corresponding 4,5-dimethoxy analogue, methylenedioxy compound 148b appears to generate a small amount of unidentified photoproducts (B(i)) in addition to the SIH and quinolin-2-one.

For aminocinnamoyl-caged PIH compounds 150a-d, decaging profiles are shown below, where it can be seen that all CICs undergo total photolysis (Figure 4.6-4.9, B) to release PIH (C) and a second signal which, for compounds 150b-c is seen to correspond to the expected carbostyril (D/E). With the 4,5-dimethoxy analogue 150a there appears to be a significant level of unexpected photoproduct appearance following irradiation of the CIC (Figure 4.6, B). This is also observed with the 4,5-methylenedioxy analogue 150b, but to a lesser extent (Figure 4.7, B).

Figure 4.5. HPLC chromatograms of 4,5-methylenedioxyaminocinnamoyl-SIH derivative 148b, showing the intact CIC (A) and the UVA (250 kJ/m²) irradiated compound (B (i)), which is magnified in B (ii) for higher resolution. The ‘naked’ SIH molecule 10 (C) and corresponding quinolin-2-one photoproduct 148b (D) are shown for reference, along with a co-injection of the irradiated CIC and SIH (E).
Figure 4.6. HPLC chromatograms of 4,5-dimethoxyaminocinnamoyl-PIH (150a), showing the intact CIC (A) and the UVA (250 kJ/m²) irradiated compound (B). The corresponding quinolin-2-one photoproduct 140a (C) is shown for reference.

Figure 4.7. HPLC chromatograms of 4,5-methylenedioxyaminocinnamoyl-PIH (150b), showing the intact CIC (A) and the UVA (250 kJ/m²) irradiated compound (B). The ‘naked’ PIH molecule 8 (C) and the corresponding quinolin-2-one photoproduct 140b (D) are shown for reference, along with co-injections of the irradiated CIC with PIH or the quinolin-2-one (E or F respectively).
Figure 4.8. HPLC chromatograms of aminocinnamoyl-PIH (150c), showing the intact CIC (A) and the UVA (250 kJ/m²) irradiated compound (B). The 'naked' PIH molecule 8 (C) and corresponding quinolin-2-one photoproduct 140c (D) are shown for reference, along with a co-injection of the irradiated CIC and the quinolin-2-one (E).
The trifluoromethyl aminocinnamate derivative 150d (Figure 4.9) undergoes clean and complete photocleavage at 250 kJ/m² (Figure 4.9, B), to release PIH 8 (R_t = 4.3 min, Figure 4.9, C) and a second photoproduct which is anticipated to be the corresponding 3-methylquinolin-2-one.

Figure 4.9.

HPLC chromatograms of 4-trifluoromethylaminocinnamoyl-PIH (150d), showing the intact CIC (A) and the UVA (250 kJ/m²) irradiated compound (B). The ‘naked’ PIH molecule 8 (C) along with co-injections of the irradiated CIC and PIH is also shown (D).
4.3.4. Various dose UVA decaging

The decaging profile of two aminocinnamoyl-CICs were evaluated with different doses of UVA radiation, as conducted previously. These included SIH derivative 148a and its corresponding PIH derivative 150a (Figures 4.10 and 4.11 respectively).

Figure 4.10. HPLC chromatograms of 4,5-dimethoxyaminocinnamoyl-SIH (148a), showing the intact CIC (A) and the UVA irradiated compound at 5 kJ/m² (B), 10 kJ/m² (C), 20 kJ/m² (D), 50 kJ/m² (E) 100 kJ/m² (F), 250 kJ/m² (G) and 500 kJ/m² (H).
Figure 4.11. HPLC chromatograms of 4,5-dimethoxyaminocinnamoyl-PIH (150a), showing the intact CIC (A) and the UVA irradiated compound at 5 kJ/m² (B), 10 kJ/m² (C), 20 kJ/m² (D), 50 kJ/m² (E) 100 kJ/m² (F), 250 kJ/m² (G) and 500 kJ/m² (H).
These results are summarised in Figure 4.12, where it is shown that the SIH derivative 148a undergoes more extensive photocleavage at lower UVA doses than its PIH counterpart 150a, which, interestingly is consistent with the photolysis of the hydroxycinnamoyl-caged compounds.

This behaviour however cannot be explained when considered in conjunction with the UV absorption spectra of these compounds. The SIH and PIH derivatives both have very similar absorption profiles, with respective $\lambda_{\text{max}}$ values of 298 and 299 nm, and exhibit analogous absorption at wavelengths between 320 and 370 nm.

Despite the considerable level of side-product generation observed in both SIH and PIH derivatives at a UVA dose of 500 kJ/m$^2$, it should be noted that such a dose of UVA is not considered to be physiologically relevant, and would be reflective of an unusually high level of solar radiation exposure. Furthermore, it is unlikely that CICs would be exposed to this level of radiation in vitro, as the level of UVA reaching intracellular compartments is reduced by endogenous biological chromophores within cellular systems.
4.4. Biological results

4.4.1. Toxicity of aminocinnamoyl-caged SIH derivatives by MTT assay

The effects of aminocinnamoyl-caged SIH compounds 148a-b on HaCaT cell growth, as measured by MTT assay are shown below (Figures 4.13 and 4.14 respectively). Cells were left untreated (control), or incubated with either SIH, the CIC or the UVA-irradiated (250 kJ/m²) CIC, and enzyme activity measured after incubation times of 24, 48 or 72 h.

![Figure 4.13. MTT assay: toxicity of aminocinnamoyl-CIC 148a on HaCaT cells (20 μM) after incubation for 24, 48 or 72 h (n = 2).](image)

According to Figure 4.13, the CIC 148a appears to be mildly toxic to the HaCaT cell line, with a degree of cell killing only apparent after 48 h, where around 20% of cellular enzymatic activity is abolished; furthermore there appears to be no increase in cell toxicity between 48 and 72 h. Thus the caged compound is considerably less toxic than the parent SIH iron chelator as one would anticipate; however cells treated with a UV-irradiated sample of 148a show no difference from the untreated controls, suggesting that the photoproducts of 148a that result from UVA-induced uncaging are non-toxic to the cells. Assuming that complete uncaging of 148a is taking place as shown in Figure 4.6 (see section 4.3), one could postulate from these results that the quinolin-2-one fragment 140a, released from CIC 148a upon UVA-irradiation somehow attenuates the toxic effect elicited by the SIH iron chelator.
A similar result was seen with the corresponding 4,5-methylenedioxy analogue 148b, (Figure 4.14) where once again the caged compound is significantly less toxic than the parent SIH iron chelator at a concentration of 20 μM; however a stronger time-dependent toxicity is observed, and the extent of cell death at 72 h is higher by approximately 8% compared to 148a, showing a slightly higher toxicity for 148b. Cells treated with the irradiated CIC, as before, show similar enzymatic activity to the untreated controls, suggesting that the corresponding quinolin-2-one fragment 148c negates the toxic effect of SIH.

Figure 4.14. MTT assay: toxicity of aminocinnamoyl-CIC 148b on HaCaT cells (20 μM) after incubation for 24, 48 or 72 h (n = 2).
4.4.2. Photoprotective effects of aminocinnamoyl-caged SIH derivatives by MTT assay

Building on these results, further experiments were conducted to assess whether the aminocinnamoyl-CICs 148a-b would protect FEK4 cells from UVA-induced damage, as these cells are considerably more susceptible to UVA damage than HaCaT cells. FEK4 cells were left untreated (control) or treated with either SIH or CIC (148a or 148b). All cells were then irradiated with UVA at a dose of either 250 kJ/m² or 500 kJ/m² and cellular enzymatic activity measured 30 min post-UVA exposure (Figure 4.15).

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 4.15.** MTT assay: toxicity of aminocinnamoyl-CICs 148a (A) and 148b (B) on FEK4 cells (20 μM). Corresponding treatments with SIH 8 (IC) are shown for reference. (n=1)
These preliminary results suggest that both CICs are non-toxic to FEK4 cells, and provide considerable protection against cellular UVA damage at 250 kJ/m². The 4,5-methylenedioxy analogue 148b however also appears to provide the same level of photoprotection at 500 kJ/m².

4.4.3. Annexin V / PI dual staining assay: aminocinnamoyl-caged SIH derivatives

The results are shown below in Figure 4.16, where cells treated with either SIH or 148a show a significantly lower rate of cell death compared to untreated cells. The degree of protection conferred by SIH is slightly higher than with the CIC, although this difference is not significant, with 84% or 91% photoprotection observed with 148a and SIH respectively at this dose of UVA. There is no significant difference between 4 h or 24 h post-UVA. It is clear from both experiments that the degree of photoprotection provided by 135a is considerably higher at 250 kJ/m² compared to 500 kJ/m².

![Figure 4.16](image_url)

**Figure 4.16.** Annexin V / PI dual staining assay: photoprotective effect of the aminocinnamoyl-caged IC 148a (20 µM), measured 4 or 24 h following irradiation of FEK4 cells at a dose of 500 kJ/m² (n = 2).
4.4.4. Reactive oxygen species (ROS) measurement assays

To further elucidate the apparent photoprotective effect seen with \(148a\) in FEK4 cells, the effect of \(148a\) as scavenger of ROS was evaluated. This would help identify whether the photoprotective effect seen by \(148a\) is due to antioxidant properties of the photoproducts, by negating the deleterious iron-mediated effects of ROS. This assay utilises CM-H\(_2\)DCFDA, \(166a\) (Scheme 4.10) an indicator which, upon entering cells undergoes cleavage at its acetate groups by cellular esterases and is then oxidised by ROS to fluorescent indicator (\(166b\)). This level of fluorescence observed is proportional to the concentration of intracellular ROS (e.g. H\(_2\)O\(_2\))\(^{[177]}\).

![Scheme 4.10. Structure of CM-H\(_2\)DCFDA (166a), and its in vitro transformation to the fluorescent species 166b by intracellular ROS.](image)

The results of the ROS assay are shown in Figure 4.17, where it is apparent that cells treated with \(148a\) have a lower intracellular ROS concentration compared to the corresponding untreated controls at doses of UVA up to 250 kJ/m\(^2\). At 500 kJ/m\(^2\) UVA however this effect is diminished, and the antioxidant capacity of the compound at this high dose of UVA radiation appears to be significantly lower. This can be explained however by the fact that cell membrane integrity is damaged at this level of UVA exposure, resulting in leakage of the fluorescent marker from the cells and the apparent decrease observed.
Figure 4.17. ROS measurement in FEK4 cells, which were either left untreated or incubated with 148 (20 μM) and then UVA-irradiated. Measurements were taken 2 h post-UVA radiation (n = 2).
4.4.5. *Annexin V / PI dual staining assay: aminocinnamoyl-caged PIH derivatives*

Following on from these results, the corresponding PIH analogue of \textit{148a}, namely, \textit{150a}, was also evaluated for photoprotective properties in FEK4 cells. The degree of photoprotection was measured by the Annexin V / PI assay following UVA irradiation. The results are shown in Figure 4.18.

The aminocinnamoyl-caged PIH compound \textit{150a} has no significant toxicity on FEK4 cells, and following UVA irradiation appears to provide a significant level of protection against UVA-induced cell death; however the extent of photoprotection conferred by CIC \textit{150a} does not appear to be more significant than that elicited by PIH alone.

Figure 4.19 shows the same experiment with the corresponding 4,5-methylenedioxy analogue \textit{150b}, and immediately it is evident that this minor structural change has a drastic effect on the biological properties of this compound. CIC \textit{150b} appears to potentiate the effects of UVA radiation on FEK4 cells treated with this compound, with approximately 80% cell killing taking place, compared to 57% cell death in untreated cells exposed to the same dose of UVA.
As the photoprotective effects of PIH have already been documented, this result may be attributable to the corresponding 3-methyl-quinolin-2-one \textit{140b}, as its photorelease from this CIC has already been demonstrated (section 4.3). Thus, it can be postulated from these results that the quinolin-2-one \textit{140b} may act as a photosensitiser which intensifies the deleterious effects of UVA radiation on these cells.

The photosensitizer properties of psoralen, \textit{22a} and its related derivatives (Figure 4.20) are well established;\textsuperscript{[94]} however more recently their pro-oxidant effects have been elucidated, and have been shown to promote the formation of ROS within cells.\textsuperscript{[178]} Given the structural similarity between the tricyclic systems of carbostyril \textit{140b} and psoralen, one could certainly anticipate that \textit{140b} is capable of pro-oxidant activity.
4.5. Future work

Investigating the photoprotective effects of the quinolin-2-one photoproducts 140a-b would allow for further clarification of the biological effects of the corresponding CICs in UVA-exposed cells. The UV-absorption spectra of carbostyrils 140a-b (Figure 4.1) show that these compounds have $\lambda_{\text{max}}$ values at 341 nm and 343 nm respectively, within the UVA range. The photoprotective effect observed with CIC 148a, which releases quinolin-2-one 140a (Figure 4.21) upon decaging may therefore, in part, be because 140a acts as a UV filter as well as an antioxidant; however this fails to explain the effects seen with 140b, which instead appears to encourage UVA-mediated cell damage.

![Figure 4.21. 3-methyl-quinolin-2-one photoproducts released from CICs 148a/150a and 148b/150b.](image)

Further work would also focus on the synthesis and evaluation of aminocinnamates without methyl substitution on the olefinic carbon, (Figure 4.22), as it has been shown this can alter the photochemical properties of the caged-compounds, such as the rate of photocleavage. This may have important implications on the applications of photoprotective CICs, and thus merits further investigation.

![Figure 4.22. Backbone of aminocinnamoyl-caged SIH without methyl substitution on the carbon-carbon double bond.](image)

It is also anticipated that, with the synthetic strategies optimised herein, a library of carbostyril analogues could be produced and evaluated for antioxidant and
photoprotective activity within relevant cell lines using the assays described. This could then allow for the rational design and preparation of the corresponding aminocinnamoyl-CICs, which could serve as multifunctional photoprotectants with potent antioxidant activity.