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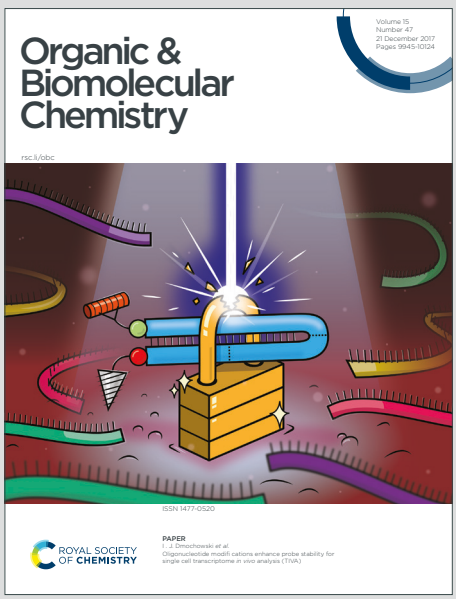

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ARTICLE

Synthesis and Evaluation of 4-(α-Arylvinyl)pyridines as Activated Linker Groups for Application in Antibody-Drug Conjugates

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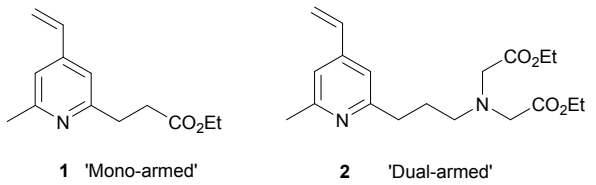
With the aim of increasing the cysteine-selective bioconjugation reactivity of 4-vinylpyridines, a series of 4-(α-arylvinyl)pyridines were synthesised by Suzuki-Miyaura cross-coupling of 4-(α-bromovinyl)pyridine. The rate of thia-Michael addition of cysteine-containing glutathione correlated with σ_x of the aryl substituent (ρ = +0.94). Introduction of electron-donating and electron-withdrawing substituents at the 3-position of 2,6-dimethyl-4-vinylpyridine all resulted in a reduction in the rate of glutathione bioconjugation, and a shift from the optimum pK_a value of approximately 6–7 with the pK_a values determined by pH gradient NMR titration. Potential 'dual-armed' 2,6-disubstituted-4-vinylpyridine antibody-drug linker groups developed previously were modified by the α-vinyl arylation methodology. This resulted in an increase in the rate of glutathione bioconjugation of approximately an order of magnitude for the *p*-nitrophenyl derivatives, providing opportunities for the development of new antibody-drug conjugates.

Introduction

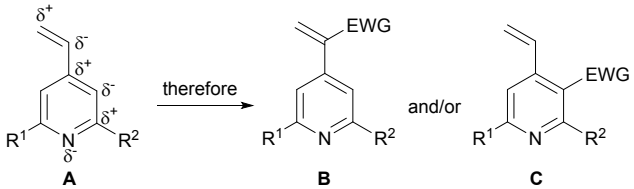
Antibody-drug conjugates (ADCs) are therapeutic agents combining the target-specific binding of an antibody with a potent cytotoxic payload.[1] Both components are covalently attached to a linker group, and for the antibody this typically exploits chemoselective bioconjugation of a nucleophilic cysteine-derived thiol with an electrophilic Michael acceptor.[2] Maleimide is used as the latter in several clinically approved ADCs, but these are prone to disassembly by retro-Michael addition.[3] Selective cysteine-based conjugation of proteins has also been achieved by exploiting the β-vinyl carbon electrophilicity of 2- and 4-vinylpyridines,[4] and ester functionalised 4-vinylpyridine **1** (Figure 1) is an ADC linker significantly less prone to reversible thiol addition.[5,6] This intrinsic stability is often a desirable feature for the design of ADC therapeutics, as deconjugation of the potent cytotoxic drug during plasma circulation can increase systemic toxicity and reduce on-target exposure leading to a narrower therapeutic index.[7,8] Related vinylheterocycles investigated for bioconjugation include vinylpyrimidines,[9] divinylpyrimidines[10] and *N*-methylated 2-vinylpyridinium salts.[11]



1 'Mono-armed' **2** 'Dual-armed'

Figure 1 4-Vinylpyridine based ADC linker groups **1**[5,6] and **2**.[12]

With the objective of delivering high-DAR (drug-antibody ratio) conjugates, we recently reported a series of difunctionalised 'dual-armed' derivatives of 4-vinylpyridine, capable of linking two cytotoxic payloads per conjugation moiety.[12] Model thia-Michael bioconjugation reactions using glutathione, a cysteine-containing tripeptide, revealed that of the many 2,6-disubstituted derivatives synthesised, only diester **2** approached the reactivity of **1**.



A **B** **C**

Figure 2 The latent polarity of a 2,6-disubstituted 4-vinylpyridine (**A**) and positioning of an electron-withdrawing group (EWG) for greater β-carbon electrophilicity (**B** and **C**).

With a view to increasing the rate of bioconjugation of 'dual-armed' 4-vinylpyridine-based linkers, we reasoned that the latent polarity of the vinylpyridine core (**A** - Figure 2) pointed to the introduction of an electron-withdrawing group (EWG) at either the α- (**B**) or 3-position (**C**) as a means of increasing the

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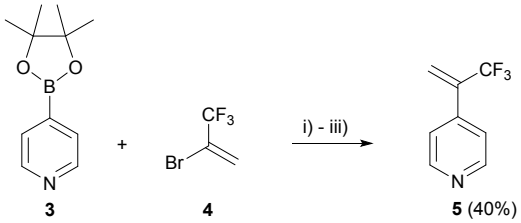
electrophilicity of the β-vinyl carbon. This paper describes our work on the synthesis and model reactivity studies of such derivatives, leading to the identification of an optimised 'dual-armed' and activated 4- vinylpyridine ADC linker.

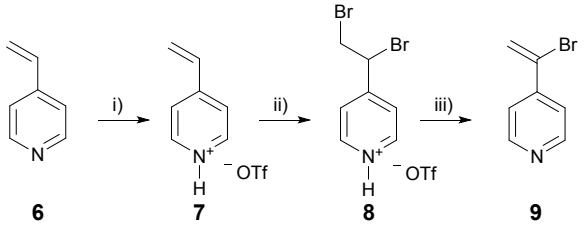
Results and discussion

Alkenes containing electron-withdrawing substituents are prone to polymerisation,[13] and at the start of this programme it was conspicuous that 4-(α-nitrovinyl)pyridine was not known, and there was only a single, characterisation free, report of 4-(α-trifluoromethylvinyl)pyridine **5**.[14] We obtained a low yield of **5** by palladium catalysed cross-coupling of 4-pyridineboronic acid pinacol ester **3** and 2-bromo-3,3,3-trifluoropropene **4** (Scheme 1),[15] followed by pyridinium salt precipitation rather than chromatography as a means of purification. Following liberation of **5** with aqueous sodium hydrogen carbonate, it was found to be highly prone to decomposition on storage, rendering analysis difficult and further testing impractical.



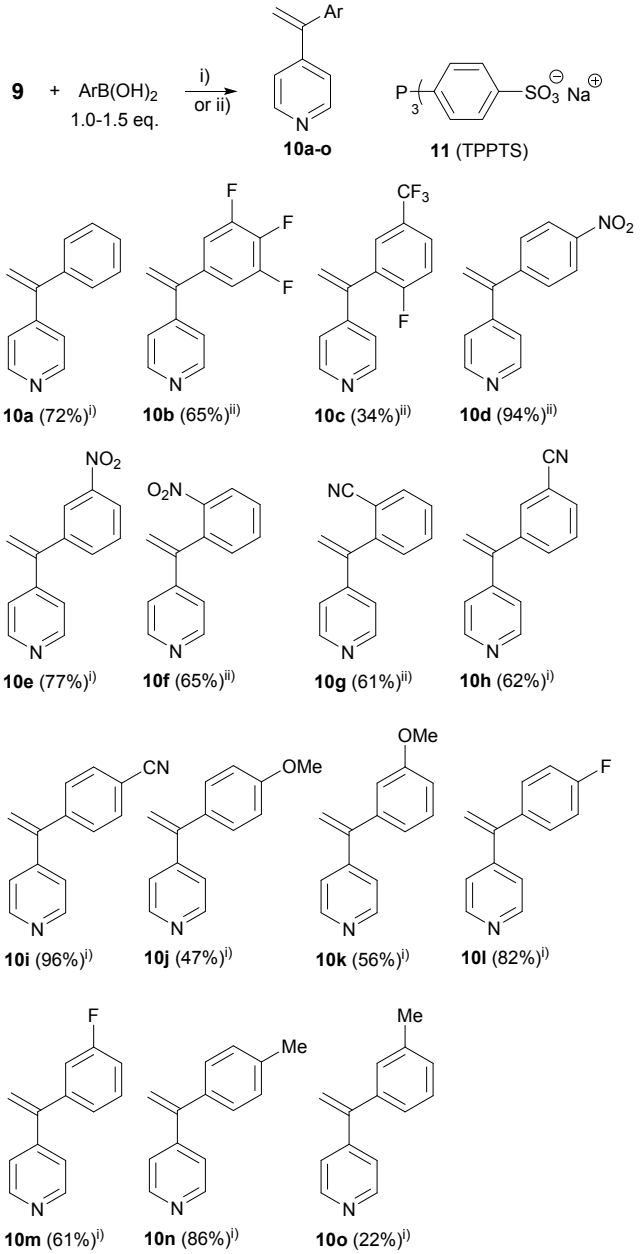
Scheme 1 Synthesis of 4-(α-trifluoromethylvinyl)pyridine **3**. i) $PdCl_2$(dppf) (10 mol%), Cs_2CO_3 (3 eq.), DME/H_2O, 80 °C, 8h. ii) 2M HCl/Et_2O – filtration. iii) Aq. $NaHCO_3$ – extraction with CH_2Cl_2.

As an alternative to the direct attachment of a strong electron-withdrawing substituent, we reasoned that introduction of an α-aryl group would enable tuning of the reactivity profile of the 4-vinylpyridine component by variation of one or more aryl substituents. Although various methodologies for the synthesis of 4-(α-aryllvinyl)pyridines have been reported,[16] palladium-catalysed cross-coupling appeared to offer the simplest approach. To this end we first synthesised 4-(α-bromovinyl)pyridine **9** in 64% yield overall as previously reported (Scheme 2).[17] As found for **5**, pyridine **9** also proved to be highly reactive, decomposing rapidly as evidenced by the formation of a black solid when left to stand either in solution or neat at -20 °C in the dark. To minimise this **9** was used in a subsequent reaction immediately following generation.



Scheme 2 Synthesis of 4-(α-bromovinyl)pyridine **9**.[17] i) TfOH (1.1 eq.), Et_2O, 0 °C, 1 h. (quant.). ii) Br_2 (2 eq.), $CHCl_3$, 0 °C to RT, 2 h. (86%). iii) NEt_3 (3 eq.), MeCN, 0 °C to RT, 3 h. (85%).

Two methods of palladium catalysis were used; starting either with $Pd(OAc)_2$ and employing the water soluble triarylphosphine ligand TPPTS **11** [conditions i) – Scheme 3], or with $Pd(PPh_3)_4$ [conditions ii)]. The former aided the isolation of the potentially polymerisation-sensitive cross-coupled product without the use of column-chromatography, although all of **10a**-**10o**, containing both electron-withdrawing and electron-donating substituents, were sufficiently stable to enable full-characterisation.



Scheme 3 Synthesis of 4-(α-arylvinyl)pyridines **10a**-**10o**. i) $Pd(OAc)_2$ (3-5 mol%), **11** (6-10 mol%), K_2CO_3 (3 eq.), 1 : 1 dioxane/H_2O, 90 °C, 16 h. ii) $Pd(PPh_3)_4$ (10 mol%), K_2CO_3 (3 eq.), 5 : 1 dioxane/H_2O, 90 °C, 16 h.

With these compounds in hand we then determined the rate of glutathione (GSH) addition to **10a** and all of the *meta* and *para* mono-substituted aryl derivatives (excepting **10m** - Scheme 4). As previously developed,[12] this model

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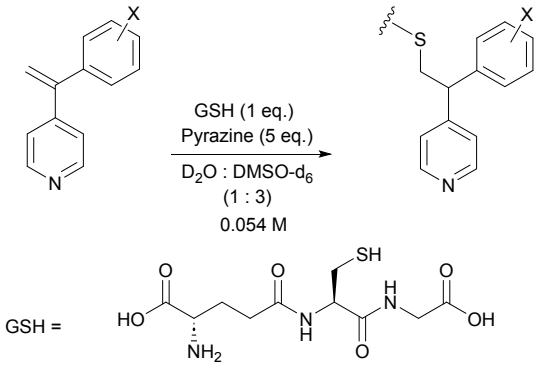
bioconjugation reaction[18] utilised one equivalent of the cysteine- containing tripeptide as a solution in D_2O (0.22 M), added to the vinylpyridine in DMSO-d_6 (0.073 M). Pyrazine was included as an internal standard for integration with the reaction monitored using ^1H NMR spectroscopy. The rates of this second order addition reaction[10,11] are given in Table 1.



Scheme 4 Reaction of 4-(α-arylvinyl)pyridines with glutathione (GSH) for the determination of rate as a function of the position and identity of the substituent X.

Table 1. Rate of reaction of selected 4-(α-arylvinyl)pyridines **10** with glutathione.

Entry	Substrate (X)	k_{obs} / M^{-1} min^{-1}	Entry	Substrate (X)	k_{obs} / M^{-1} min^{-1}
1	**10a** (H)	0.088	2	**10d** (*p*-NO_2)	0.480
3	**10e** (*m*-NO_2)	0.245	4	**10h** (*m*-CN)	0.230
5	**10i** (*p*-CN)	0.577	6	**10j** (*p*-OMe)	0.033
7	**10k** (*m*-OMe)	0.080	8	**10l** (*p*-F)	0.127
9	**10n** (*p*-Me)	0.065	10	**10o** (*m*-Me)	0.086

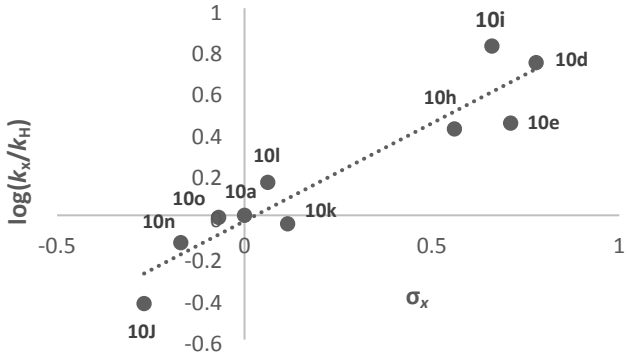
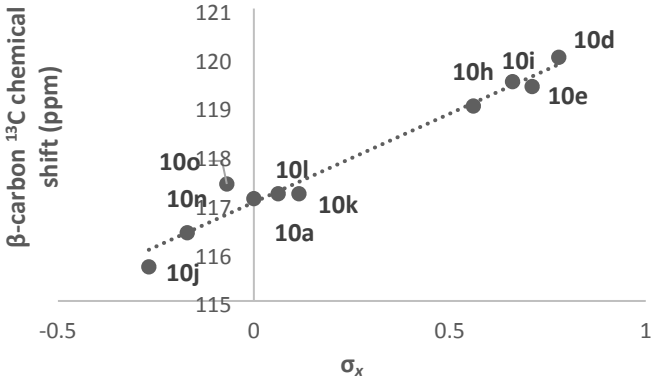


Figure 3 Plot of $\log(k_x/k_H)$ *vs.* Hammett substituent constant (σ_x) for glutathione addition to 4-(α-arylvinyl)pyridines **10** (ρ = 0.94, R^2 = 0.89)

For comparison, the rate of addition of GSH to 4-vinylpyridine was determined as 0.14 M^{-1} min^{-1}. Thus the introduction of an α-phenyl substituent is deactivating (entry 1, **10a**), but this can be more than compensated for by the introduction of a *para*-nitro (**10d**, entry 2) or *para*-cyano (**10l**, entry 5) substituent, with the latter giving an approximately four-fold increase in rate over 4-vinylpyridine. A plot of $\log(k_x/k_H)$ *vs.* Hammett substituent constant (σ_x) further confirmed the influence of a substituent's identity and position on reactivity (Figure 3), and the value of the reaction constant (ρ = +0.94) is consistent with increased electron-density in the transition state of rate-determining S-C bond forming conjugate addition. Furthermore, the relative electrophilicity of a substrate, as measured by β-carbon ^{13}C NMR chemical shift,[19] correlates with σ_x (Figure 4) and by extension with the rate of glutathione addition.[20]

Figure 4 Plot of β-carbon ^{13}C chemical shift vs. Hammett substituent constant (σ_x) 4-(α-arylvinyl)pyridines **10** (R^2 = 0.97)



We next examined the influence of a substituent at the 3-position, and for this study it was convenient to start with 4-bromo-2,6-dimethylpyridine **11a** due to the simplicity of mono-nitration to give **11b** (Scheme 5). Subsequent reduction to **11c** was followed by diazotisation/fluorination, methylation or acetylation to give **11d**, **11e** and **11f** respectively. Subsequent Suzuki-Miyaura cross-coupling with potassium vinyltrifluoroborate[12] gave vinylpyridines **12a**-**12f**. The rate of GSH addition to each of these was determined as before (Table 2).

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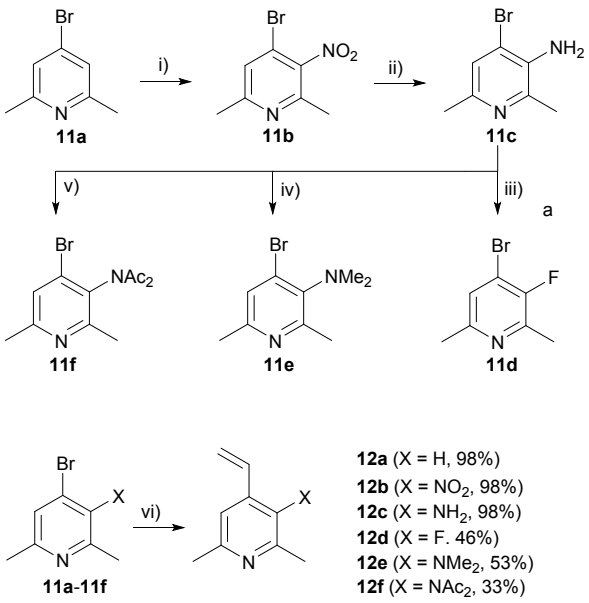

Scheme 5 Synthesis of 4-vinyl-2,6-dimethylpyridine **12a** and 3-substituted derivatives **12b-f**. i) HNO_3 (3 eq.), H_2SO_4, 100 °C, 16 h. (40%). ii) Fe (12 eq.), EtOH, $HCl_{(aq)}$, Δ, 2 h. (99%). iii) a) HBF_4 (2 eq.), $NaNO_2$ (1 eq.), H_2O, 0 °C, b) PhMe, Δ, 16 h. (34%). iv) 37% CH_2O (2.5 eq.), HCO_2H (6.5 eq.), Δ, 16 h (46%). (v) Ac_2O (1.2 eq.), AcCl (2.6 eq.), NEt_3 (2.2 eq.), CH_2Cl_2, RT, 2 d. (59%). vi) Potassium vinyltrifluoroborate (2 eq.), NEt_3 (3 eq.), $PdCl_2$(dppf) (5 mol%), 2 : 1 PhMe/*i*-PrOH, 90 °C.

Table 2 Rate of GSH addition, ^{13}C NMR and pK_a analysis of vinylpyridines **12a-f**.

Entry	Substrate (X)	k_{obs} / M^{-1} min^{-1}	β-carbon ^{13}C (ppm)[a]	pK_a
1	**12a** (H)	1.2	118.2	7.03 +/- 0.08
2	**12b** (NO_2)	0.073	122.7	2.83 +/- 0.04
3	**12c** (NH_2)	0.49	119.1	7.44 +/- 0.07
4	**12d** (F)	0.28	120.5	ND[b]
5	**12e** (NMe_2)	0.3	117.3	7.30+/- 0.07
6	**12f** (NAc_2)	0.2	121.8	ND[c]

[a]$CDCl_3$. [b]Not determined due to instability. [c]Not determined due to poor aqueous solubility (see ESI).

The value of k_{obs} (M^{-1} min^{-1}) obtained for **12a** (entry 1, X = H) is similar to the values reported previously for 2,6-disubstituted vinylpyridines **1** (0.80) and **2** (0.75). None of the 3-substituted derivatives improved the rate, and the reactivity of **12b** containing the strongly electron-withdrawing nitro-substituent was much reduced. As for the 4-(α-arylvinyl)pyridines **10** discussed above, the β-carbon ^{13}C chemical shifts (Table 2) increase in value with the increasing electron-withdrawing nature of the substituent X.[21] However, in contrast to the α-(4-nitrophenyl) derivative **10d**, the introduction of a nitro substituent at the 3-position to give **12b** decreases rather than increases reactivity.

To examine this further we used our previously developed pH gradient NMR titration methodology for pK_a determination (Table 2).[12] In this earlier study on 2,6-disubstituted-4-vinylpyridines a linear relationship was observed between the rate of GSH addition and pK_a within the range of 1 (low reactivity) to 6–7 (high reactivity) [for **1** (pK_a = 6.18) and **2** (pK_a = 6.59)]. With pK_a > 7 the reactivity decreased. The hypothesis that pyridine basicity principally controls reactivity was rationalised by participation in rate-determining conjugate addition of the corresponding pyridinium species, with the reactivity of this protonated intermediate attenuated by electron-donating substituents (where pK_a > 7).[12] This mechanistic proposal is supported by the similar outcomes observed for the 3-substituted derivatives **12**, and in particular the decreased reactivity of **12b** as a result of the decrease in pK_a to 2.83 (Table 2, entry 2). This analysis also explains the higher reactivity of 2,6-dimethyl-4-vinylpyridine **12a** (rate = 1.2) compared to less basic 4-vinylpyridine (rate = 0.14) for which a pK_a of 5.6 has been determined.[22]

The above results revealed that improved bioconjugation reactivity in a 'dual-armed' vinylpyridine-based linker was most likely to result from the introduction of a *para*-nitrophenyl or *para*-cyanophenyl moiety at the α-position. In addition to **2**, we also applied this adaptation to 2,6-diester **13** (pK_a = 5.36, rate = 0.17 M^{-1} min^{-1}).[12] The methodology described above for the synthesis of **10** was found to be applicable to both compounds (Scheme 6). In each case the α-bromo derivative generated by elimination was not isolated but instead was used immediately in a Suzuki-Miyaura cross-coupling to give α-aryl derivatives **14**-**17**.

The determination of rates of GSH addition as before (Table 3) revealed an approximately order of magnitude increase in reactivity for the *para*-nitrophenyl containing derivatives **14** and **16** (entries 1 and 3) relative to their corresponding parents (**2** and **13** respectively). Significantly, the similarity of the pK_a values of **14** (6.21, entry 1) and **2** (6.59)[12] points to the 8-fold increase in the rate of GSH addition to the former being the result of increased β-carbon electrophilicity *without* the *para*-nitrophenyl substituent significantly attenuating pyridine basicity. This is similarly the case for **16** (pk_a 5.37, entry 3) compared to **13** (5.36).[12] The rate increases for the *para*-cyanophenyl derivatives **15** and **17** are smaller (entries 2 and 4). These differences between the influence of the *p*-nitro and *p*-cyano substituents are reflected in the higher β-carbon ^{13}C chemical shift and higher pK_a value of the compounds containing the former.

Finally, as the Hammett substituent constant (σ) for the 4-pyridyl group (0.94)[23] is similar to σ_p for the nitro group (0.78) we reasoned that 1,1-di(*para*-nitrophenyl)ethene **19**[24] may also display reactivity towards GSH addition. This is the case (entry 5), although with a rate significantly less than that of **14**. Thus the presence of strong electron-withdrawing groups in both aryl substituents of a 1,1-diarylethene results in the thia-Michael reaction of the glutathione cysteine component, a reaction accelerated further by a sufficiently basic pyridine moiety.

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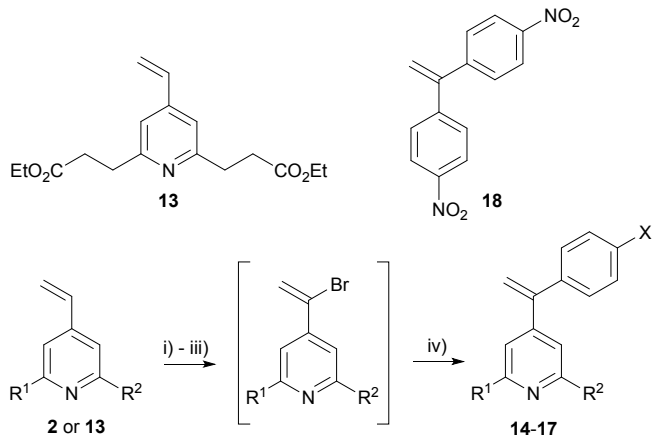

14 [R^1 = CH_3, R^2 = $(CH_2)_3N(CH_2CO_2Et)_2$, X = NO_2] 51%
15 [R^1 = CH_3, R^2 = $(CH_2)_3N(CH_2CO_2Et)_2$, X = CN] 53%
16 [R^1 = R^2 = $(CH_2)_2CO_2Et$, X = NO_2] 48%
17 [R^1 = R^2 = $(CH_2)_2CO_2Et$, X = CN] 30%

Scheme 6 Synthesis of activated functionalised 'dual-armed' 4-vinylpyridine derivatives **14**-**17**. i) TfOH (1.1 eq.), $CHCl_3$, 0 °C, 30 min. ii) Br_2 (2 eq.), $CHCl_3$, RT. iii) NEt_3 (3 eq.), MeCN, RT. iv) *p*-$XC_6H_4B(OH)_2$ (1.5 eq), $PdCl_2(PPh_3)_2$ (5 mol%), K_2CO_3 (3 eq.), 5 : 1 dioxane/H_2O, 90 °C.

Table 3 Rate, of GSH addition, ^{13}C NMR and pK_a analysis of activated functionalised 4-vinylpyridine derivatives **14**-**17** and diaryl derivative **18**.

Entry	Substrate (X)	k_{obs} (M^{-1} min^{-1})	Rate increase	β-carbon ^{13}C (ppm)[a]	pK_a[b]	pK_a change
1	**14** (NO_2)	5.8	8[c]	119.3	6.21 ± 0.03	-0.38[e]
2	**15** (CN)	2.7	4[c]	118.8	6.11 ± 0.013	-0.48[e]
3	**16** (NO_2)	2.3	14[d]	119.5	5.37 ± 0.12	0.01[f]
4	**17** (CN)	0.4	3[d]	118.8	4.79 ± 0.05	-0.57[f]
5	**18**	2.6	NA	120.2	NA	NA

[a]$CDCl_3$.. [b]Of the pyridine. moiety. The pK_a of the amine component of **15** was determined as 2.69 ± 0.05 (3.12 for **2**[12]). [c]Compared to **2** (0.75). [d]Compared to **13** (0.17). [e]Compared to **2** (6.59). [f]Compared to **13** (5.36).

Conclusions

The rate of thia-Michael addition of cysteine-containing glutathione to 4-(α-arylvinyl)pyridines, a model bioconjugation reaction, increases with the electron-withdrawing capacity of the substituted aryl group. The highest rates, which are approximately 3-4 times faster than 4-vinylpyridine, were obtained with a *p*-cyanophenyl or *p*-nitrophenyl aryl moiety. In contrast, introduction of a 3-nitro substituent to 2,6-dimethyl-4-vinylpyridine results in a significant decrease in rate. In both cases the introduction of an electron-withdrawing group increases the electrophilicity of the β-vinyl carbon of the Michael acceptor as determined by ^{13}C NMR chemical shift. However, in the latter case the introduced 3-nitro group also significantly decreases pyridine basicity, and thus the percentage of the corresponding activated pyridinium ion generated by acid catalysis. Application of the methodology developed for the synthesis of the 4-(α-arylvinyl)pyridines enabled the direct modification of previously developed 'dual-armed' 2,6-disubstituted-4-vinylpyridine ADC linkers.[12] The rate of model bioconjugation to the new 4-(α-arylvinyl) derivatives increases by up to an order of magnitude with a *p*-nitrophenyl group. These results provide opportunities for protein bioconjugation validation as the next step in the development of new ADCs.

Author contributions

TES, GRS, MW and CJR conceived the project. JA and JMS prepared the compounds and performed the kinetic analysis under the supervision of JT, PLKH, GRS, CJR and TES. MW designed and performed the pH gradient NMR titration experiments. CJR wrote the manuscript, with suggestions from all other authors.

Conflicts of interest

There are no conflicts to declare.

Data availability

Additional data supporting this article (synthetic details, other experimental procedures, NMR spectra) are available in the ESI[+].

Acknowledgements

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21 For **12a**-**f** a plot of β-carbon ^{13}C chemical shift *vs*. σ_m gives R^2 = 0.88 and *vs*. σ_p gives R^2 = 0.74 (see ESI). These data highlight the relationship between the electronic properties of the 3-substituent and the β-vinyl carbon ^{13}C chemical shift, but being *ortho* steric influences may also be significant.

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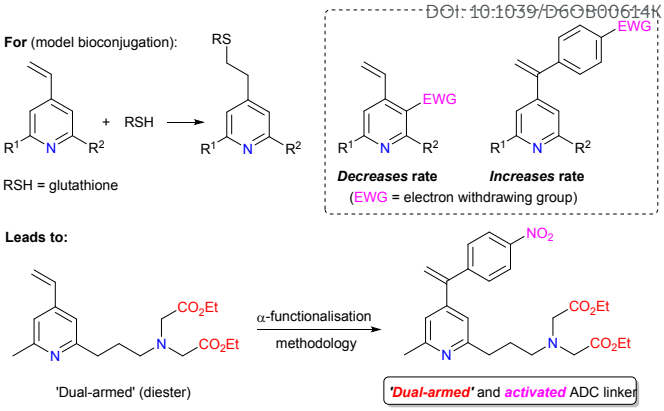

The rate of thia-Michael addition of glutathione to 2,6-disubstituted-4-vinylpyridines increased by approximately an order of magnitude by introduction of an α-(4-nitrophenyl) substituent.

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Data availability

Additional data supporting this article (synthetic details, other experimental procedures, NMR spectra) are available in the ESI†.