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Isothiocyanatoporphyrins, useful intermediates for the conjugation of porphyrins with biomolecules and solid supports

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***meso*-Phenylporphyrins bearing a single isothiocyanate group react efficiently under ambient conditions with primary amino groups on proteins and polystyrene resins.**

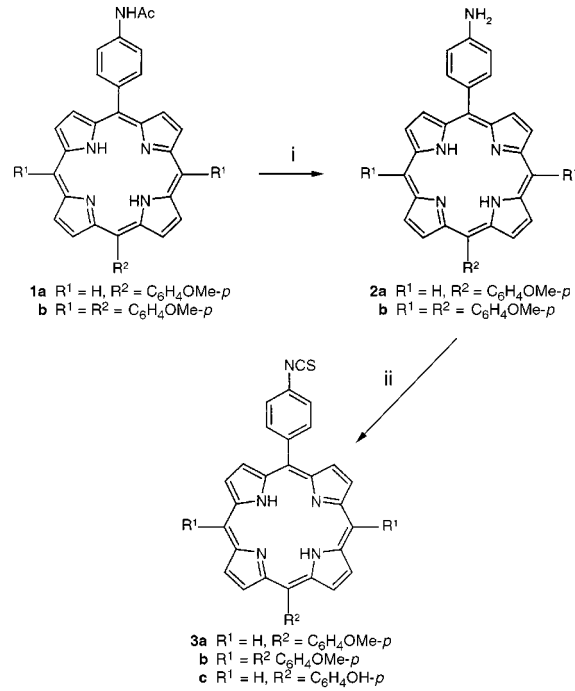
Porphyrins are molecules of great interest due to the wide range of potential applications associated with them, these include photochemotherapy,[1] oxidation catalysis,[2] optoelectronics[3] and fluorescence imaging.[4] In order to customise porphyrins for uses such as these it is often necessary to attach the core macrocycle to either biological targeting agents, such as proteins, or solid supports. The conjugation of porphyrins with these species is often complicated by solvent incompatibilities and/or the forcing conditions commonly required to react substituents on the porphyrin periphery. We have recently become interested in developing mild methods for the conjugation of porphyrins and report here a facile method for generating a single isothiocyanate group on a porphyrin and the reaction of these species with amines in solution, on polypeptides and on polystyrene resins.

Previous attempts have been made to conjugate porphyrinoid structures with a variety of biologically active macromolecules including lipoproteins,[5] monoclonal antibodies[6] and serum albumin.[7] Many of these complexes rely on non-covalent linkages which, while more easily formed, are inherently less stable than covalent conjugates. Immunoconjugates[8] have been produced *via* non-covalent interaction of a porphyrin hapten at specific binding sites present on the surface of antibodies. Such porphyrin immunoconjugates have been used to confer specificity to photochemotheraputic techniques but the preparation and stability of the conjugate is heavily dependent on conditions such as solvent, pH and temperature. Clearly a covalently bound porphyrin antibody conjugate would be more flexible in terms of the physical and chemical conditions it could be exposed to. The majority of attempts to covalently conjugate porphyrin molecules with polypeptides have relied on carbodiimide mediated coupling to protein amino residues, *via* carboxy functionality on the macrocycle. These reactions are however slow and require the *in situ* generation of a reactive intermediate, which can lead to extensive loss of porphyrin as the *N*-acyl urea derivative.

Isothiocyanates are commonly used for the attachment of fluorescent probes to sensitive biological substrates under very mild conditions with no by-products,[9] however the use of this type of functionality in porphyrin chemistry has, strangely, not been developed. Collman[10] recently reported the use of porphyrin isocyanates as molecular building blocks for the construction of capped and strapped porphyrins. The high reactivity of isocyanates makes them useful synthetic tools, but their use is complicated by rapid hydrolysis of the NCO group upon exposure to moisture. Currently this synthesis involves reaction of amino porphyrins with highly toxic phosgene or triphosgene under inert atmosphere conditions. Isothiocyanates in contrast are slightly less reactive, but considerably more stable, their hydrolysis being much slower under aqueous conditions. Here we present a convenient method for synthesising porphyrin isothiocyanates and demonstrate their use in porphyrin conjugation with amino bearing substrates.

Acid hydrolysis of mono acetamido porphyrins, synthesised according to Adler *et al.*[11] and Boyle *et al.*,[12] yielded the corresponding mono amino derivatives. Subsequent treatment with 1,1′-thiocarbonyldi-2,2′-pyridone (TDP)[13] in CH_2Cl_2 at room temperature (Scheme 1) gave, quantitatively, the respective monoisothiocyanato porphyrins after 30 to 40 min. The by-product, 2-hydroxypyridine, was easily removed during an aqueous work up. The resulting monoisothiocyanato porphyrins are relatively stable to air and can be handled without the need for inert atmosphere conditions. In order to test the reactivity of these compounds a number of reactions were performed under standard conditions, in solution, using a variety of amines; these results are summarised in Table 1.

It can be seen from these results that the isothiocyanate functionality reacts cleanly and in good to excellent yield with most amines. Primary aliphatic amines were found to react fastest with times typically in the region of 30 min. Aromatic and secondary amines react more slowly and the combination of these structural features in the same molecule (*e.g.* diphenylamine) resulted in no detectable product being formed, even after 10 days. *N*-Terminal amino residues of phenylalanine, leucine and valine were also found to react cleanly. All the resulting thioureidyl porphyrin derivatives showed good stability, including those formed from amino acids. We believed that this was important with regard to the use of monoisothiocyanato porphyrins for bioconjugation with polypeptides.



Scheme 1 *Reagents and conditions*: i, 6 M HCl, 100 °C, 3 h, 10% Et_3N–CH_2Cl_2, aqueous workup; ii, 1,1′-thiocarbonyldi-2,2′-pyridone (2 equiv.), CH_2Cl_2, 25 °C, 0.5–1 h, aqueous workup. Porphyrins **1a** to **3c** characterised by ^1H and ^{13}C NMR, MS and UV-VIS spectroscopy.

Table 1 Reactions of monoisothiocyanato porphyrin **3a** with selected amines and amino acids

Entry	Substrate	*t*/h	Yield[a] (%)
1	Allylamine	1	85
2	Benzylamine	2	89
3	Butylamine	0.5	95
4	Cyclohexylamine	1	92
5	L-Phe-OMe-HCl	17	95
6	L-Leu-OMe-HCl	30	91
7	L-Val-OMe-HCl	17	92

[a] Isolated thiourea derivatives characterised by ^{1}H and ^{13}C NMR, MS and UV–VIS.

Table 2 Reactions of monoisothiocyanato porphyrin **3b** with selected amines and amino acids

Entry	Substrate	*t*/h	Yield[a] (%)
1	Allylamine	1	95
2	Aniline	120	65
3	Benzylamine	2	70
4	Butylamine	0.5	98
5	Cyclohexylamine	1	87
6	Isopropylamine	0.5	93
7	Propargylamine	2	64
8	Dibenzylamine	3	78
9	Dicyclohexylamine	2	94
10	Diethylamine	1	92
11	Diphenylamine	240	0[b]
12	L-Phe-OMe-HCl	21	89
13	L-Leu-OMe-HCl	36	94
14	L-Val-OMe-HCl	24	94

[a] Isolated thiourea derivatives characterised by ^{1}H and ^{13}C NMR, MS and UV–VIS. [b] No detectable reaction.

Encouraged by these results we set out to explore the potential of monoisothiocyanato porphyrins for bioconjugation and attachment to polystyrene resins.[14] Bovine and human serum albumin (BSA and HSA) are well-characterised proteins which have been used in drug delivery[15] and for raising antibodies by haptenisation of small molecules. We therefore selected BSA as a model protein to study the potential of monoisothiocyanato porphyrins for bioconjugation. Model reactions on amines with monoisothiocyanato porphyrins had previously been performed in organic solvents; in order to facilitate conjugation with proteins under aqueous conditions, a 5,15-diphenylporphyrin was synthesised bearing a more hydrophilic hydroxy group on one phenyl ring and an isothiocyanate group on the opposing phenyl ring[12] (Scheme 1, **3c**).

In a typical experiment a stock solution of 5-(4-hydroxyphenyl)-15-(4-isothiocyanatophenyl)porphyrin **3c** in anhydrous DMSO (10 mg ml^{-1}) was prepared. 100 μl of the stock solution was added to a gently stirred solution of BSA (10 mg) in carbonate buffer (1 ml, pH 9.2). The reaction was protected from light, and agitated at room temperature for 17 h. Purification of the conjugate was achieved using gel filtration (Sephadex G-25) eluting with carbonate buffer at pH 8.9. The porphyrin–BSA conjugate was contained in the first fluorescent band eluted from the column, and was characterised by SDS-PAGE. Once the gel had been run to completion (200 mA, 45 min) the unstained gel plates were illuminated with a UV lamp revealing a red fluorescent band which, on subsequent staining with silver nitrate, corresponded with BSA at 66 kD in the protein reference ladder.

The UV–VIS spectrum of the red fluorescent material at 66 kD exhibited both the characteristic Soret and Q bands of the porphyrin and an absorption peak at 284 nm corresponding to BSA. The same material showed strong porphyrin fluorescence at 620 nm. Standard spectroscopic methods[16] were used to determine the labelling ratio of the porphyrin–BSA conjugate, which was found to be 1 : 1.

Finally, monoisothiocyanato porphyrin was reacted with Rink amide resin (Novabiochem). Pre-swelling of the resin in CH_2Cl_2 followed by Fmoc deprotection of the amino group in 20% piperidine–DMF, then washing with MeOH, CH_2Cl_2, DMF and water yielded the free amino Rink resin. Monoisothiocyanato porphyrin (3 equiv.) was then mixed with the resin (1 equiv.) in CH_2Cl_2 and agitated for 17 h at 25 °C in the dark. The loading value for monoisothiocyanato porphyrin on the Rink resin was calculated at 0.718 mmol g^{-1}, representing a loading efficiency of 94.1%. Treatment with 1% TFA–CH_2Cl_2 solution for 17 h resulted in quantitative cleavage of porphyrin from the resin as the thiourea derivative. Interestingly, cleavage conditions for Rink amide resin usually require at least 50% TFA solutions.[17]

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