S(*O*)-Pixyl protecting group as efficient mass-tag†

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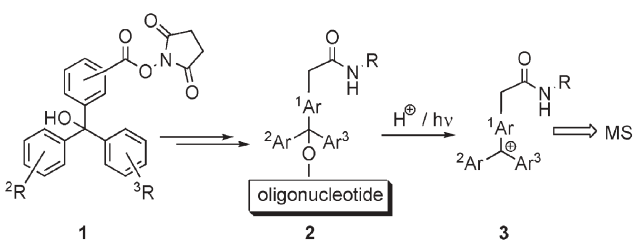
We report herein the design, preparation and first applications of novel trityl tags with adjustable stability, efficient as protecting groups or MS analytes.

Mass spectrometry (MS) plays a major role in the study of the genome[1] and the proteome[2] but there is still room for improvement. In particular, methods that increase sensitivity and dynamic range would represent a significant advance. In this context, chemical tagging strategies have been developed to overcome the limits of analysing native molecular species.[3]

As a result of their stability as carbocations, triarylmethyl derivatives (trityls) produce exceptional mass spectra under (MA)LDI conditions (laser desorption ionisation, both with and without matrix), and can be detected down to *atto*mole levels.[4] Additionally, trityls show a remarkable structural malleability. For instance, bifunctional trityls have been used in areas such as bioconjugation, cross-linking, optics *etc*.[4] We aimed to combine these properties to give rise to a range of new molecular tools with applications in MS analysis.

When used as labels for genome analysis, trityl mass-tags are typically attached to oligonucleotides through an ether bond. A wide range of masses is accessible from a precursor **1** by treating a NHS (*N*-hydroxy succinimide) activated side-chain with different amines. In use, tags are released by acid or UV irradiation as cations **3** for MS analysis (Scheme 1).[5]



Scheme 1 Trityl-based mass tags.

We have shown that the more stable a trityl cation is, the better its detection in MS. However, ether bonds linking trityl tags that produce the most stable cations are too labile to survive post-labelling protocols. An ideal trityl mass-tag should be stable as a protecting group (trityl ether) but, upon request, generate a stable cation. We show here that these antagonist characteristics can be combined into a single trityl structure, stable as a protecting group but, also, capable of generating efficient mass tags either upon chemical treatment or under LDI MS conditions.

† Electronic supplementary information (ESI) available: Experimental details. See http://www.rsc.org/suppdata/cc/b5/b504913j/

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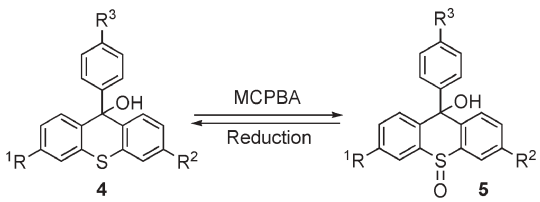
Trityls based on the 9-arylthioxanthenyl skeleton (*S*-pixyl)[6] **5c** proved to be suitable candidates.‡ *S*-pixyl cations are notably stable and, as it happens with the triphenylmethyl series, can be further stabilised by the addition of methoxy substituents to the position 3 and/or 6 of the thioxanthenyl moiety,§ as was confirmed by pK_{R+} measurements (Table 1).

The properties of the *S*-pixyl derivatives were dramatically modified when the sulfur atom was oxidised to the corresponding sulfoxide (Scheme 2, Table 1). Resulting *S*(*O*)-pixyl compounds were resistant to acid treatment and were not ionised even in 50% H_2SO_4. The reverse deoxygenation of the sulfoxide function in *S*(*O*)-pixyl derivatives was satisfactorily carried out according to literature procedures.[7]



Scheme 2 Interconversion of *S*-pixyls.

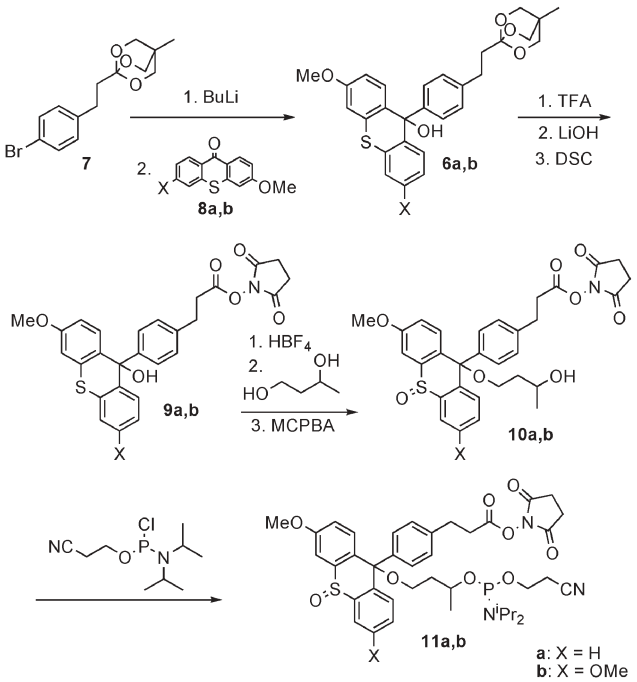
Table 1 pK_{R+} of *S*-pixyl cations

			pK_{R+}[a]			
R^1	R^2	R^3	S-Px		S(O)-Px	
H	H	Me	**4a**	−0.2	**5a**	<−15
H	H	OMe	**4b**	0.2	**5b**	≈−11
OMe	H	Me	**4c**	2.8	**5c**	≈−12
OMe	OMe	Me	**4d**	4.5	**5d**	−7.1

[a] pK_{R+} determined spectrophotochemically.[8]

Encouraged by our initial findings, we moved on to the preparation of bifunctional *S*-pixyl mass-tags, equipped with a NHS activated side-chain and a phosphoramidite reactive centre to attach the tag to oligonucleotides.

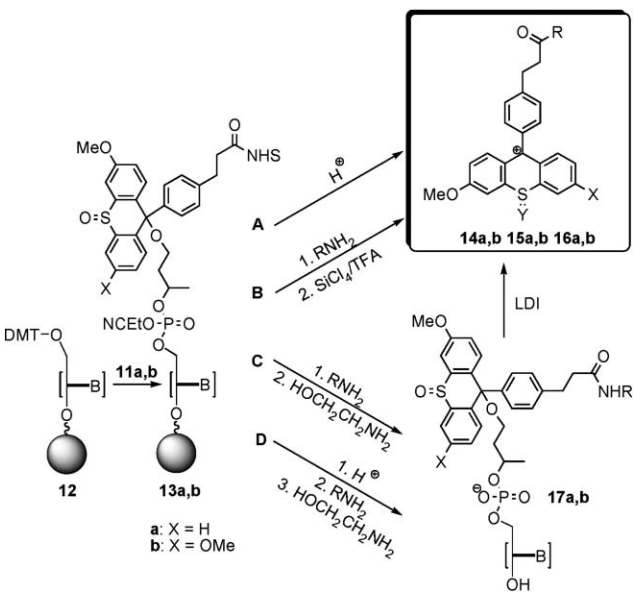
S-pixyls **6**, containing a carboxylic acid protected as an *ortho* ester,[9] were prepared by the reaction of the lithium derivative of **7** with 3-methoxythioxanthen-9-ones **8** (Scheme 3). The *ortho* esters were then converted into the NHS activated esters with good yield. The selective tritylation of the primary hydroxy group of 1,3-butanediol was completed *via* the tetrafluoroborate salt of compounds **9**.[10] *In situ* treatment with MCPBA yielded the *stable* version of the mass-tags, *S*(*O*)-pixyls **10**. Phosphitylation of the secondary hydroxyl group yielded the desired functionalised tags **11**. The phosphoramidite function did not show compatibility problems with the presence of the sulfoxide in the molecule.



Scheme 3 Synthesis of *S*(*O*)-pixyl mass tags.

For evaluation as mass tags, *S*(*O*)-pixyls **11** were incorporated into the 5′-end of model oligonucleotides **12** using a standard protocol on an automated DNA synthesiser (Scheme 4).

A series of tests and experiments were designed to evaluate the scope of the novel *S*(*O*)-pixyl tags. We aimed to determine their properties as protecting groups followed by the optimisation of their use as mass tags. Oligonucleotides protected with NHS functionalised *S*(*O*)-pixyl tags were treated with amines and so different mass tags were generated. Table 2 lists the possible ions shown in Scheme 4: ions **14** would retain the sulfoxide function whereas ions **15** and **16** would be in the corresponding sulfide



Scheme 4 From the oligonucleotide synthesis to the mass spectrometer.

Table 2 Molecular weight of ions **14**, **15** and **16** in Scheme 4

Compound	Y	X	R	MW
14a	O	H	NHS	472.12
14b	O	OMe	NHS	502.13
15a	—	H	NHC_4H_{10}	430.18
15b	—	OMe	NHC_4H_{10}	460.19
16a	—	H	NHC_5H_{12}	444.20
16b	—	OMe	NHC_5H_{12}	474.21

form; ions **15** and **16** are differentiated by the alkyl chain of the amide function.

The acid stability of the 5′ protecting group in oligonucleotides **13** was assessed using standard deblock solution (path A, Scheme 4). Oligonucleotide **13a** remained protected whereas oligonucleotide **13b** cleaved to yield **14b**.

We then tested the chemical activation of the *stable* mass-tags. After coupling **13** with butyl amine, CPG resins were treated with a 0.1 M solution of $SiCl_4$/TFA in dichloromethane producing an instantaneous orange colour in the flow-through solution, characteristic of the presence of the *S*-pixyl cation (path B, Scheme 4). The generation of the *S*-pixyl cation could be explained by the initial reduction of the sulfoxide function to the corresponding sulfide by $SiCl_4$ and subsequent generation of the *S*-pixyl cation under reductive-acidolysis conditions.[7] (MA)LDI analysis of the flow-through solution confirmed the presence of tagged systems **15**.

S(*O*)-pixyl tags were exposed to the conditions required for the liberation of oligonucleotides and the deprotection of the phosphate groups. Resins **13a,b** were coupled with different amines, depending on whether they were exposed to acid or not; systems that were not treated with acid were reacted with butylamine (path C, Scheme 4) and systems treated with acid were reacted with pentylamine (path D, Scheme 4). Oligonucleotides **17a,b** were deprotected and cleaved off the resin by treatment with ethanolamine. A fraction of the obtained solutions were purified by HPLC, confirming the stability of the *S*(*O*)-pixyl tags to the experimental conditions.

Our efforts were then directed to finding the best conditions for the MS analysis of labelled oligonucleotides **17a,b** through the incorporated *S*-pixyl mass tags. For the chemical activation of the mass-tags, a range of reagents for the deoxygenation of sulfoxides in aqueous media was evaluated, among them $Na_2S_2O_4$, $Na_2S_2O_5$ and HI.[11] The (MA)LDI analysis of the different crude mixtures revealed the presence of the tags **15** and **16**, with cleaner spectra when the analysis was carried out without matrix (under LDI conditions). This fact confirms the exceptional ability of the *S*-pixyl tags to produce MS spectra under LDI conditions, with no other peaks detectable.

To our surprise, we found that the best analytical results were obtained when the oligonucleotide solutions were directly spotted in the MS plate, without any pre-treatment to release the tags and analysed under LDI conditions, regardless of whether they had been purified by HPLC, eliminating the possibility of false positives due to left over tags.

Despite analysing the non-purified reaction mixtures in no-matrix conditions, we obtained clean MS spectra containing exclusively the signals of the *S*-pixyl mass-tags; peaks related to oligonucleotides were not detected. This was a remarkable finding, since it effectively eliminated the cleanup and sample preparation

steps prior to performing the MS analysis, which are regarded as weaknesses in MALDI MS analyses.[12]

For instance, Fig. 1 shows the MS spectra obtained under these conditions of a mixture of oligonucleotides **17a** (0.5 μl; 1e–4 mM), obtained from **13a** through paths C and D in Scheme 4. The spectrum shows the peaks corresponding to ions **15a** and **16a**, Table 2.

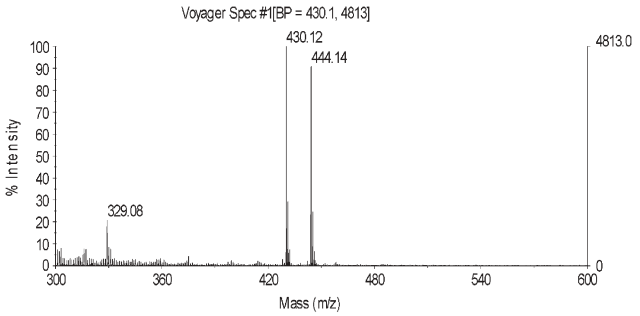


Fig. 1 MS LDI spectrum of an equimolar mixture of oligonucleotides **17a** (R = NHC_4H_{10} and NHC_5H_{12}): only mass tags are detected.

These results show that the oxidised *S*(*O*)-pixyl tags are reduced and cleaved by the laser in the mass spectrometer. A literature review revealed that certain sulfoxides can be converted to their corresponding sulfides by direct photolysis in the presence of sensitizers,[13] and that *S*-pixyl protected nucleosides can be deprotected when irradiated at 300 nm.[14] Thus, these two processes occur simultaneously under LDI analytical conditons.

Confirming our previous findings, LDI MS analysis of an equimolar mixture of oligonucleotides protected with monomethoxy *S*(*O*)-pixyl and dimethoxy *S*(*O*)-pixyl (oligonucleotides **17a** and **17b** through path C, Scheme 4) showed the correlation between the stability of the corresponding *S*-pixyl cations and the intensity of the peaks they yield in MS (Fig. 2, peaks corresponding to ions **15a** and **15b** in Table 2). Despite the lower acid stability of the dimethoxy *S*(*O*)-pixyl tags, they could be used when higher degree of MS sensitivity is required.

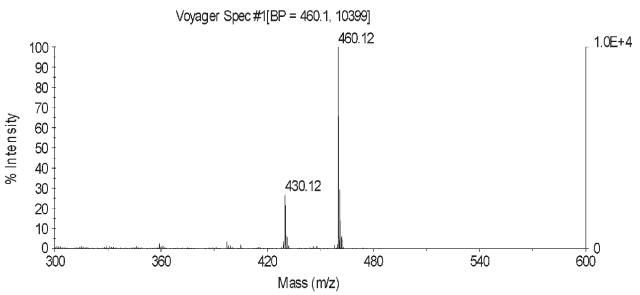


Fig. 2 MS LDI spectrum of an equimolar mixture of oligonucleotides **17a** and **17b**; R = NHC_4H_{10}.

In this communication we have introduced the concept of trityls with adjustable stability with applications in mass spectrometry. Using the *S*-pixyl skeleton as a starting framework and taking benefit from the redox chemistry and photochemistry of its sulfur atom and the unique properties of the *S*-pixyl group as a whole, we have prepared bifinctional trityl systems which have application as mass tags.

In addition, we believe the compounds we have described have the potential to be useful in other areas, such as protecting groups[15] or safety-catch handles for solid phase chemistry.[16] Investigations are being carried out in our laboratory to delineate the scope and limitations of our system with adjustable stability.

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Notes and references

‡ Alternative approaches using different heteroatoms (N, S, Se) and structures are included in the supplementary information.†
§ In recent work carried out in our laboratory, we have successfully developed a novel synthetic route to 3-methoxythioxanthenone and 3,6-dimethoxythioxanthenone, from which 3-methoxy and 3,6-dimethoxy substituted *S*-pixyl derivatives are readily available. Submitted for publication.

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