

Reactive trityl derivatives: stabilised carbocation mass-tags for life sciences applications†

Alexey V. Ustinov,^a Vadim V. Shmanai,^b Kaajal Patel,^c Irina A. Stepanova,^a Igor A. Prokhorenko,^a Irina V. Astakhova,^a Andrei D. Malakhov,^{‡a} Mikhail V. Skorobogaty,^a Pablo L. Bernad Jr,^c Safraz Khan,^c Mona Shahgholi,^d Edwin M. Southern,^e Vladimir A. Korshun^{*a} and Mikhail S. Shchepinov^{*c}

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The rational design of novel triarylmethyl (trityl)-based mass tags (MT) for mass-spectrometric (MS) applications is described. We propose a “ pK_{R^+} rule” to correlate the stability of trityl carbocations with their MS performance: trityls with higher pK_{R^+} values ionise and desorb better. Trityl blocks were synthesised that have high pK_{R^+} values and are stable in conditions of MS analysis; these MTs can be ionised by matrix as well as irradiation with a 337 nm nitrogen laser. ¹³C-Labelled tags were prepared for MS quantitation applications. Moreover, the tags were equipped with a variety of functional groups allowing conjugation with different functionalities within (bio)molecules to enhance the MS characteristics of the latter. The MS behaviour of model polycationic trityl compounds with and without the matrix was studied to reveal that poly-trityl clusters are always singly charged under the (MA)LDI-TOF conditions. Several peptide-trityl conjugates were prepared and comparisons revealed a beneficial effect of trityl tags on the conjugate detection in MS. Trityl compounds containing *para*-methoxy- and dimethylamine groups, as well as a xanthene fragment, showed considerable enhancement in MS detection of model peptides; thus they are promising tools for proteomic applications. Dimethoxytrityl derivatives allow one to distinguish between Arg- and Lys-containing peptides. Maleimido trityl derivatives are suitable for the efficient derivatisation of thiol-containing peptides in pyridine.

Introduction

The life sciences are increasingly reliant on MS as a universal tool of unmatched versatility.¹ MS techniques are employed in the “omics” sciences – genomics,² glycomics,³ metabolomics⁴ and other areas, and are particularly successful in top-down and bottom-up proteomics approaches, which study complex mixtures of proteins and peptides.^{1c-e,5} Despite the progress, many issues remain to be improved, such as sample separation, sensitivity, dynamic range, quantitation etc. Ultimately, enhancing the MS ionisation and desorption ability of peptides would still be a highly desired development.

Trityl (triarylmethyl) derivatives easily ionise, desorb, and can be efficiently detected by MS techniques due to stability of corresponding carbocations.⁶ Importantly, ionisation is a result of a laser irradiation and does not require the assistance of a matrix. This has been put to a practical use by employing trityl mass-tags (MT) in combinatorial chemistry⁶ and life sciences areas such as genomics⁷ and proteomics⁸ in combination with (MA)LDI-TOF detection.

Other MT strategies, most notably quaternary amine⁹ and triphenylphosphonium-based derivatives,^{9b,10} have been developed and compared.¹¹ The charge on these moieties is permanent (in contrast with trityl derivatives, which acquire the charge upon acidic treatment or irradiation with light). This substantially limits the applications, since it is easier to control the non-quaternary charge on a trityl through substituents on aromatic rings and steric factors than to control/regulate the quaternary charge on nitrogen or phosphorus atoms. In addition, the cleavable bond between the heteroatom and the α -carbon (Scheme 1), prior to generation of the cation is a useful tool, which is unavailable with quaternary MTs.

Existing applications can be improved, and new applications for life sciences area and beyond could emerge, provided the trityl core structure is further optimised and equipped with suitable functionalities. Mass spectra of a number of trityls detectable with similar efficiency can be used for precise calibration of mass spectrometers. Furthermore, combinations of trityls with different masses yield spectra resembling bar-coding patterns. This, and the

^aShemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Miklukho-Maklaya 16/10, Moscow, 117997, Russia. E-mail: korshun@mail.ibch.ru; Fax: +7 495 3306738; Tel: +7 495 3306738

^bInstitute of Physical Organic Chemistry, Surganova 13, Minsk, 220072, Belarus

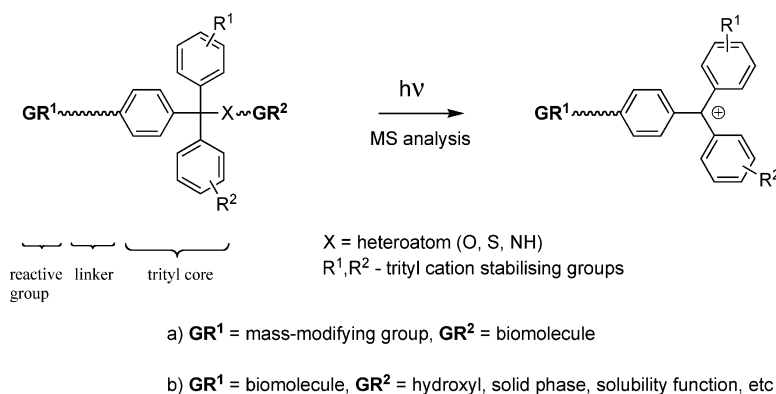
^cTridend Technologies (a Division of OGT), Sandy Lane, Yarnton, Oxford, OX5 1PF, UK. E-mail: misha@tridend.com; Fax: +44 1865 842116; Tel: +44 7766058079

^dDepartment of Chemistry, Caltech, Pasadena, CA, 91125, USA

^eOGT IP, Ltd, Begbroke Business & Science Park, Sandy Lane, Yarnton, Oxford, OX5 1PF, UK

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‡ Present address: Department of Chemistry, Texas A & M University, College Station, TX 77842, USA.



Scheme 1 Design of mass-tagging reagents: a) cleavable mass tag, b) covalently attached mass tag.

fact the trityls can be detected at very low concentrations, indicates a potential use of these compounds as security and anti-counterfeit labels. Yet more applications are possible if similarly ionisable trityls with different masses are temporarily attached to some target (bio)molecules or a solid phase through a central atom. One trityl block can be converted into an equally efficiently detectable set of compounds with different masses by taking advantage of, for example, commercially available amines,⁶ and activated ester coupling chemistry^{7b,8b} (variations in GR¹, case (a) on Scheme 1).

A mass-tagging tool should contain a reactive group (GR) for attachment to a substrate such as a (bio)molecule; a linker connecting this group to a trityl (with both the linker and the position of attachment being important in the MT performance); and a trityl, capable of producing a stable carbocation prior or during the MS analysis. In this paper, we describe in detail the rational design of trityl compounds and functional moieties permanently attached to aryl groups (case (b) on Scheme 1). The structure of the part attached through an X heteroatom and bearing GR², which is disconnected from the MT when the cation is generated, has been reported elsewhere.^{7b,8b,c}

The aim of our research was to elucidate the scope and limitations of the use of easily cationised triarylmethyl derivatives as covalently attached tags/enhancers for mass spectrometry of biomolecules, by optimising different parts of a mass-tag structure and investigating the properties of corresponding conjugates. In our quest for more efficient mass-tags we tested a variety of different structures as summarised below.

Results and discussion

pK_{R+} as a measure of trityl stability

Since the trityl part of the tag is vital for the MS analysis, we will first define the physico-chemical parameter that correlates the structural features of the triarylmethyl group with its MS performance. We found that there is a correlation between the stability of triphenylmethyl carbocations and their propensity for efficient ionisation and desorption; trityls which generate more stable cations can be detected at lower concentrations. pK_{R+} is used to express the stability of (trityl) cations and to quantify the required acidic conditions to generate those cations.¹² The single most important factor influencing the stability of triarylmethyl

carbocations is the number and position of electron donating and/or cation-stabilising groups. For instance, additional methoxy groups in the *ortho* or *para* position of the trityl's phenyl rings stabilise the corresponding cation more, and consequently it is possible to prepare trityl cations stable even under neutral conditions (with pK_{R+} in excess of 7.5).¹³

The stability of trityl cations has a direct impact on the MS analysis of these systems. This is well illustrated on Fig. 1, which shows the LDI-TOF MS spectrum of an equimolar mixture of 5 different trityl alcohols. Despite the fact that the analysed sample contained the same number of molecules of each compound, there is an evident correlation between the stability of the cations and the sensitivity of the analysis; the more stable the trityl cation is, the better its detection in MS.

Trityls with high pK_{R+} preferably stay in the ionic form (as salts). As we wanted to retain the bond between an α -carbon and X (Fig. 1) for some applications, we set out to design and test trityl blocks with pK_{R+} values not exceeding 6.

Synthesis of trityl blocks

We used several approaches¹⁴ to build up a large number of functionalised trityl blocks. Scheme 2 illustrates the approach where the first step is the attachment of the linker to a benzophenone. 4-Hydroxybenzophenones **1–3** were alkylated with two *tert*-butyl ω -halogenalkanoates, Cl(CH₂)₃CO₂Bu' and Br(CH₂)₃CO₂Bu', to give ketones **4**. *tert*-Butyl esters are stable enough to allow the reaction of benzophenones **4** with arylmagnesiumhalogenides to yield tritanols **5**. The *tert*-butyl protecting group in compounds **5** was removed using treatment with CF₃CO₂H. The obtained acids **6** can be converted into three activated derivatives – oxysuccinimide esters **7**, oxysulfosuccinimide esters **8**, and pentafluorophenyl esters **9**. All these esters can react with amines to yield amides **10**.

An alternative approach is shown in Scheme 3. In this case the trityl assembly starts with the lithiation of bromo derivatives **11** carrying an orthoester-protected carboxy function, followed by reaction with various diarylketones. The orthoester protection of carboxyl is compatible with organolithium reagents, in contrast to Bu' esters. Tritanols **12** can be easily purified by column chromatography. Mild acid hydrolysis of the orthoester function gives diol esters **13**, and further alkaline hydrolysis leads to acids

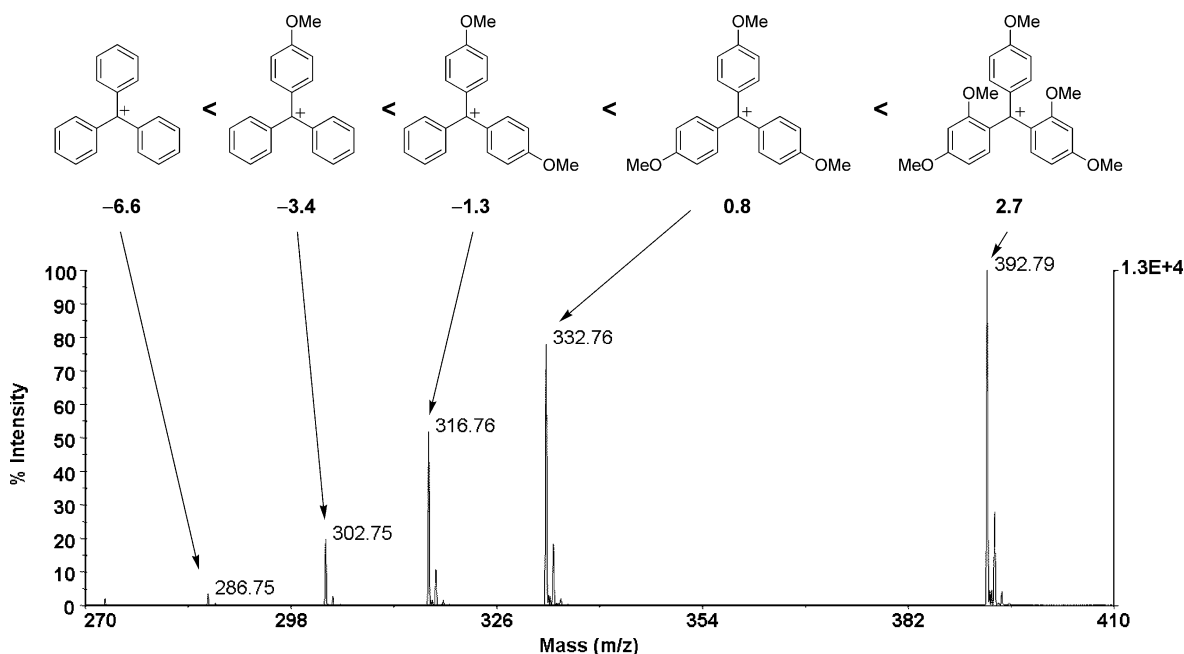


Fig. 1 LDI mass-spectrum of equimolar mixture of Ph_3COH , $4\text{-MeOC}_6\text{H}_4\text{CPh}_2\text{OH}$, $(4\text{-MeOC}_6\text{H}_4)_2\text{CPhOH}$, $(4\text{-MeOC}_6\text{H}_4)_3\text{COH}$, and $(2,4\text{-(MeO)}_2\text{C}_6\text{H}_3)_2\text{C}(4\text{-MeOC}_6\text{H}_4)\text{OH}$; no matrix.

14. The latter were converted to NSu esters **15** or water-soluble sulfoNSu esters **16**. The reaction of activated esters **15** and **16** with amines gives amides **17**.

We tested several linker systems (see Scheme 1), aiming at developing an appendage that would give high coupling yields in conjugation reactions, would be stable in MS and would not decrease the pK_{R^+} of the trityl core. Direct attachment of an activated carboxyl group to one of the phenyls of a trityl,^{6,15} either in the *para*- or the *meta*-position, led to a decrease in pK_{R^+} of a corresponding MT, which had a detrimental effect on the MS detection efficiency. We therefore attached a butyric linking moiety through an oxygen atom, thus retaining the number of alkoxy groups on the system. Several different compounds were prepared as shown above (**7a–c, h, 8**, Scheme 2; **15g–h**, Scheme 3). For applications where cross-linking using the trityl reagent would be required, or for expanding the range of trityl MTs with different masses generated from amine libraries,^{6,7a} we have also designed trityl blocks bearing two carboxy functions. These were also based on butyric linkers (**7e–f, 9**, Scheme 2).

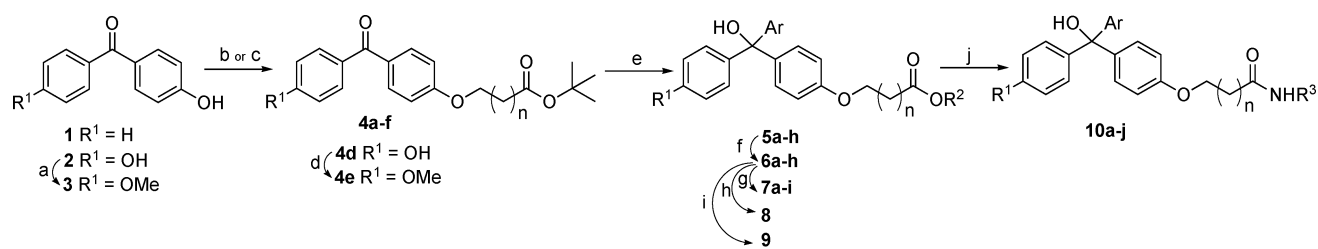
This linker was found to be unstable in conditions of (MA)LDI-TOF MS use, with carbon-oxygen bond fragmenting with release of a quinoidal trityl cation, presumably due to a favourable formation of a 5-membered ring^{8b} (MS data not shown). To avoid this, we extended the length of the linker by 2 carbons, employing the hexanoic acid based synthon (**7d, i**, Scheme 2). This linker turned out to be more stable in MS analysis, but still some of the quinoidal cleavage product was detected (MS data not shown), suggesting that a carbon-carbon bond based linker attachment would be a better choice.

This was achieved by a different strategy ($n = 0$; Scheme 3) whereby a synthon **11b** was reacted with a variety of ketones, generating trityl blocks with a very MS-stable two-carbon linker.

Xanthone and thioxanthone groups render trityl cations higher stability compared to non-bridged systems, so we prepared several trityl blocks incorporating these moieties. Stability of trityl cations (and trityl ethers) based on a thioxanthone moiety can be manipulated by converting a bridged sulfur atom into a corresponding sulfoxide, which can then be reduced back to sulfide.¹⁶

To get access to trityl blocks with three *para*-methoxy groups, we had to attach a linker through a *meta*-position and introduce a linker into a ketone. Scheme 4 shows the introduction of alkyl linker into dimethoxybenzophenone and the synthesis of several trialkoxytrityls, including one ¹³C-labelled compound. 3-Iodo-4,4'-dimethoxybenzophenone (**18**) was obtained from 3-iodo-4-methoxybenzoic acid or, more conveniently, by direct iodination of 4,4'-dimethoxybenzophenone. Compound **18** can be easily demethylated using BBr_3 in DCM to give 3-iodo-4,4'-dihydroxybenzophenone **19**. The latter was alkylated to yield diethyl ether **20** and dibenzyl ether **21**. Iodo derivatives **18**, **20**, and **21** underwent a Sonagashira-type coupling reaction with *tert*-butyl 5-hexynoate. The obtained alkynes **22** were hydrogenated over 10% Pd/C to give alkyl derivatives **23**. In the course of hydrogenolysis of compounds **22**, the alkoxy groups are cleaved and the product is the dihydroxyketone **23c**. This was alkylated with [¹³C]ethanol using Mitsunobu conditions to afford **23d**. Ketones **22a, 23a, b, d** were reacted with ArMgBr to yield corresponding tritanol derivatives **29** and **24**. These were hydrolysed to acids **30** and **25** and further converted into activated oxysuccinimide (**31** and **26**) or sulfoxysuccinimide (**27**) esters.

Isotopic labelling is widely used in MS proteomics analysis to distinguish between species with identical masses, for instance for quantitative comparison between peaks corresponding to the same peptide obtained from two different sources. Two



#	n	Ar	R ¹	R ²
4a	2		H	
4b	4		H	
4c	2		O(CH ₂) ₃ CO ₂ Bu'	
4d	2		OH	
4e	2		OMe	
4f	4		OMe	
5a	2	2-thienyl	H	Bu'
5b	2	4-C ₆ H ₄ NMe ₂	H	Bu'
5c	2	4-C ₆ H ₄ OMe	H	Bu'
5d	4	4-C ₆ H ₄ OMe	H	Bu'
5e	2	2-thienyl	O(CH ₂) ₃ CO ₂ Bu'	Bu'
5f	2	4-C ₆ H ₄ NMe ₂	O(CH ₂) ₃ CO ₂ Bu'	Bu'
5g	2	4-C ₆ H ₄ OMe	O(CH ₂) ₃ CO ₂ Bu'	Bu'
5h	2	2-thienyl	OMe	Bu'
5i	2	4-C ₆ H ₄ NMe ₂	OMe	Bu'
5j	2	4-C ₆ H ₄ OMe	OMe	Bu'
5k	4	4-C ₆ H ₄ OMe	OMe	Bu'
6a	2	2-thienyl	H	H
6b	2	4-C ₆ H ₄ NMe ₂	H	H
6c	2	4-C ₆ H ₄ OMe	H	H
6d	4	4-C ₆ H ₄ OMe	H	H
6e	2	2-thienyl	O(CH ₂) ₃ CO ₂ H	H
6f	2	4-C ₆ H ₄ NMe ₂	O(CH ₂) ₃ CO ₂ H	H
6g	2	4-C ₆ H ₄ OMe	O(CH ₂) ₃ CO ₂ H	H
6h	2	2-thienyl	OMe	H

#	n	Ar	R ¹	R ²
6i	2	4-C ₆ H ₄ NMe ₂	OMe	H
6j	2	4-C ₆ H ₄ OMe	OMe	H
6k	4	4-C ₆ H ₄ OMe	OMe	H
7a	2	2-thienyl	H	NSu
7b	2	4-C ₆ H ₄ NMe ₂	H	NSu
7c	2	4-C ₆ H ₄ OMe	H	NSu
7d	4	4-C ₆ H ₄ OMe	H	NSu
7e	2	2-thienyl	O(CH ₂) ₃ CO ₂ Su	NSu
7f	2	4-C ₆ H ₄ OMe	O(CH ₂) ₃ CO ₂ Su	NSu
7g	2	2-thienyl	OMe	NSu
7h	2	4-C ₆ H ₄ NMe ₂	OMe	NSu
7i	4	4-C ₆ H ₄ OMe	OMe	NSu
8	2	4-C ₆ H ₄ OMe	OMe	NSu-SO ₃ Na
9	2	4-C ₆ H ₄ OMe	O(CH ₂) ₃ CO ₂ C ₆ F ₅	C ₆ F ₅
#	n	Ar	R ¹	R ³
10a	2	4-C ₆ H ₄ OMe	H	(CH ₂) ₃ CH ₃
10b	2	2-thienyl	OMe	(CH ₂) ₃ CH ₃
10c	2	4-C ₆ H ₄ OMe	OMe	(CH ₂) ₄ CH ₃
10d	4	4-C ₆ H ₄ OMe	H	(CH ₂) ₃ CH ₃
10e	4	4-C ₆ H ₄ OMe	OMe	(CH ₂) ₃ CH ₃
10f	2	4-C ₆ H ₄ NMe ₂	OMe	(CH ₂) ₄ Ph
10g	2	4-C ₆ H ₄ OMe	OMe	(CH ₂) ₂ NMe ₃ ⁺
10h	2	4-C ₆ H ₄ OMe	OMe	1/2 of (CH ₂) ₂ N((CH ₂) ₂ CONH(CH ₂) ₂)
10i	2	4-C ₆ H ₄ NMe ₂	OMe	EGVNDNEGFSSAR
10j	2	4-C ₆ H ₄ NMe ₂	OMe	EGVNDNEGFSSAR

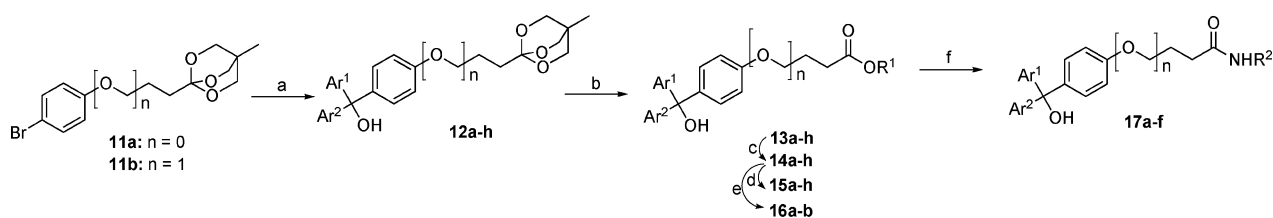
Scheme 2 Introduction of alkoxy linker into benzophenones and preparation of triarylmethanol derivatives. *Reagents and conditions*: a) MeI, NaH, DMF, rt; b) Cl(CH₂)₃CO₂Bu', MeONa, HMPA, 100 °C; c) Br(CH₂)₃CO₂Bu', K₂CO₃, acetone, rt; d) MeI, K₂CO₃, acetone, rt; e) ArMgBr, THF, rt; f) CF₃CO₂H, DCM, rt; g) DSC, Et₃N, DCM, rt; h) HONSu-SO₃Na, DCC, DMF, rt; i) C₆F₅OH, DCC, EtOAc, rt; j) R³NH₂, THF, rt.

popular techniques dubbed ICAT and iTRAQ are often employed whereby proteomes or peptidomes are labelled with two thiol-specific or amine-specific reagents identical but for their isotopic composition.¹⁷ To make the trityl MT labelling platform compatible with this isotope-based quantification method, we prepared a series of compounds identical in structure but different in mass. A deuterium-based approach is easier to implement as one simply needs to reduce triple bonds with D₂ to arrive at a set of compounds different from the hydrogen reduced ones by 4 Da. Unfortunately, deuterated compounds are eluted differently from hydrogenated ones on HPLC, making it difficult to follow LC-MS proteomics protocols. To obviate this problem, we used ¹³C labelling when preparing ketone building blocks **23d** for trityl assembly. Target compound **27b**, when used in combination with a non-labelled version **26c**, gives a pair of chemically and structurally identical MTs with 4 Da mass difference. Importantly, this scheme allows one to prepare MTs with only one R¹ group containing the

heavier version of ethyl, giving rise to a series of 3 MTs with 2 Da difference. This is useful if more than 2 conjugates need to be quantified by MS. Trityl chemistry permits for even more isotopically labelled alkyl groups to be introduced should the need arise. Importantly, unlike the ICAT reagents, the trityl MTs are "active" in that they not only serve as mass appendages to differentiate between analyte molecules, but also enhance the MS performance of the conjugates (*vide infra*, Fig. 8, 9). Work is now in progress to employ these trityl tags in quantitation of peptides by MS.

To be able to combine a xanthyl or a thioxanthyl moiety with an aryl block to which no side-arm could be (easily) attached, *e.g.* a pyrenyl¹⁶ residue, we developed an approach allowing for a linker to be connected to a (thio)xanthyl block. Synthesis of an alkyl functionalised xanthone trityl is shown on Scheme 5.

3-Hydroxy-6-methoxy-9*H*-xanthen-9-one (**33**) was converted into the phenol triflate in good yield. Our strategy to convert



#	n	Ar ¹	Ar ²	R ¹
12a	0	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	
12b	0			
12c	0	4-C ₆ H ₄ SMe	4-C ₆ H ₄ SMe	
12d	0			
12e	0			
12f	0			
12g	1	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	
12h	1	4-C ₆ H ₄ SMe	4-C ₆ H ₄ SMe	
13a	0	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	CH ₂ CMe(CH ₂ OH) ₂
13b	0			CH ₂ CMe(CH ₂ OH) ₂
13c	0	4-C ₆ H ₄ SMe	4-C ₆ H ₄ SMe	CH ₂ CMe(CH ₂ OH) ₂
13d	0			CH ₂ CMe(CH ₂ OH) ₂
13e	0			CH ₂ CMe(CH ₂ OH) ₂
13f	0			CH ₂ CMe(CH ₂ OH) ₂
13g	1	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	CH ₂ CMe(CH ₂ OH) ₂
13h	1	4-C ₆ H ₄ SMe	4-C ₆ H ₄ SMe	CH ₂ CMe(CH ₂ OH) ₂
14a	0	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	H
14b	0			H
14c	0	4-C ₆ H ₄ SMe	4-C ₆ H ₄ SMe	H

#	n	Ar ¹	Ar ²	R ¹
14d	0			H
14e	0			H
14f	0			H
14g	1	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	H
14h	1	4-C ₆ H ₄ SMe	4-C ₆ H ₄ SMe	H
15a	0	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	NSu
15b	0			NSu
15c	0	4-C ₆ H ₄ SMe	4-C ₆ H ₄ SMe	NSu
15d	0			NSu
15e	0			NSu
15f	0			NSu
15g	1	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	NSu
15h	1	4-C ₆ H ₄ SMe	4-C ₆ H ₄ SMe	NSu
16a	0	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	NSu-SO ₃ Na
16b	0	4-C ₆ H ₄ SMe	4-C ₆ H ₄ SMe	NSu-SO ₃ Na
#	n	Ar ¹	Ar ²	R ²
17a	0	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	(CH ₂) ₄ CH ₃
17b	0	4-C ₆ H ₄ SMe	4-C ₆ H ₄ SMe	(CH ₂) ₄ CH ₃
17c	0	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	1/2 of -CH ₂ CH ₂ -
17d	0	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	1/3 of C ₆ H ₃ -1,3,5-(CONH(CH ₂) ₃) ₃
17e	0	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	EGVNDNEGFFSAK
17f	0	4-C ₆ H ₄ OMe	4-C ₆ H ₄ OMe	EGVNDNEGFFSAR

Scheme 3 Alkyl or alkoxy linker on Grignard reagent: preparation of triarylmethanol derivatives. *Reagents and conditions:* a) 1) BuLi, THF, -70 °C, 2) Ar¹COAr², THF, -40 °C; b) CF₃CO₂H, H₂O, THF; c) 1) NaOH, EtOH, H₂O; 2) H⁺; d) DSC, Et₃N, DCM, rt; e) HONSu-SO₃Na, DCC, DMF, rt; f) R²NH₂, THF, rt.

the triflate **34** into **35** via a Sonogashira palladium cross-coupling reaction with *tert*-butyl hex-5-ynoate proceeded as anticipated, allowing a facile way of introducing the linker side arm. Grignard addition of 4-methoxyphenyl magnesium bromide to compound **35** yielded tritolan **36**, and further synthetic steps finally gave the desired compounds **40**. Hydrogenation of compound **38** was triple bond specific and did not affect the trityl carbinol.

Maleimide is a convenient thiol-reactive function for the labelling of cysteine-containing proteins and peptides. In the preparation of maleimide derivatives of tritolans (Schemes 6 and 7) a hydroxyl group was used as a precursor for a maleimide. Tetrahydropyranyl protection was used for the OH group. Bromoaryl precursor **41** was lithiated and then reacted with 4,4'-dimethoxybenzophenone. The acidic removal of the Thp group gives trityl methyl ether **43**. This reacted with maleimide under Mitsunobu conditions. Toluene was found to be the solvent of

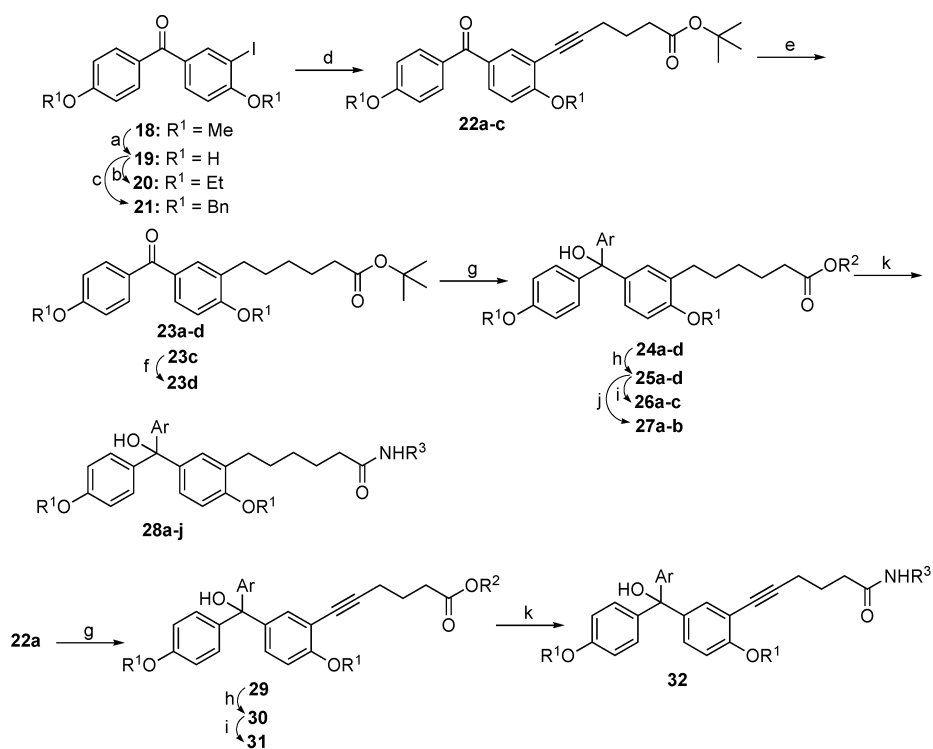
choice for this reaction. The careful removal of Me group gives tritolan **45** (Scheme 6).

The maleimide derivative of trimethoxytritolan (Scheme 7) was prepared using synthetic strategies shown on Schemes 4 and 6.

We have thus prepared a variety of trityl blocks with different pK_{R+} values bearing an assortment of reactive groups (oxysuccinimide, sulfo-oxysuccinimide, and pentafluorophenyl esters or maleimido) for testing in conjugation reactions.

Charge state of trityl clusters

We anticipated that conjugation of mass-tags with polyfunctional molecules such as peptides could result in multiple labelling of, say, several amino groups. To investigate the effect of mass-tagging, including multiple attachments of mass-tags (possibly resulting in formation of multiply charged ions), on MS behaviour, we



#	R ¹	Ar	R ²
22a	Me		
22b	Et		
22c	Bn		
23a	Me		
23b	Et		
23c	H		
23d	[¹³ C]Et		
24a	Me	4-C ₆ H ₄ OMe	Bu'
24b	Me	4-C ₆ H ₄ NMe ₂	Bu'
24c	Et	4-C ₆ H ₄ OMe	Bu'
24d	[¹³ C]Et	4-C ₆ H ₄ OMe	Bu'
25a	Me	4-C ₆ H ₄ OMe	H
25b	Me	4-C ₆ H ₄ NMe ₂	H
25c	Et	4-C ₆ H ₄ OMe	H
25d	[¹³ C]Et	4-C ₆ H ₄ OMe	H
26a	Me	4-C ₆ H ₄ OMe	NSu
26b	Me	4-C ₆ H ₄ NMe ₂	NSu
26c	Et	4-C ₆ H ₄ OMe	NSu
27a	Me	4-C ₆ H ₄ OMe	NSu-SO ₃ Na
27b	[¹³ C]Et	4-C ₆ H ₄ OMe	NSu-SO ₃ Na

#	R ¹	Ar	R ³
28a	Me	4-C ₆ H ₄ OMe	(CH ₂) ₃ CH ₃
28b	Me	4-C ₆ H ₄ OMe	(CH ₂) ₄ CH ₃
28c	Me	4-C ₆ H ₄ OMe	(CH ₂) ₅ CH ₃
28d	Me	4-C ₆ H ₄ OMe	(CH ₂) ₆ CH ₃
28e	Me	4-C ₆ H ₄ OMe	(CH ₂) ₈ CH ₃
28f	Me	4-C ₆ H ₄ OMe	(CH ₂) ₉ CH ₃
28g	Me	4-C ₆ H ₄ OMe	(CH ₂) ₂ Ph
28h	Me	4-C ₆ H ₄ OMe	(CH ₂) ₃ Ph
28i	Me	4-C ₆ H ₄ OMe	EGVNDNEEGFFSAK
28j	Me	4-C ₆ H ₄ OMe	EGVNDNEEGFFSAR

#	R ¹	Ar	R ²
29	Me	4-C ₆ H ₄ OMe	Bu'
30	Me	4-C ₆ H ₄ OMe	H
31	Me	4-C ₆ H ₄ OMe	NSu

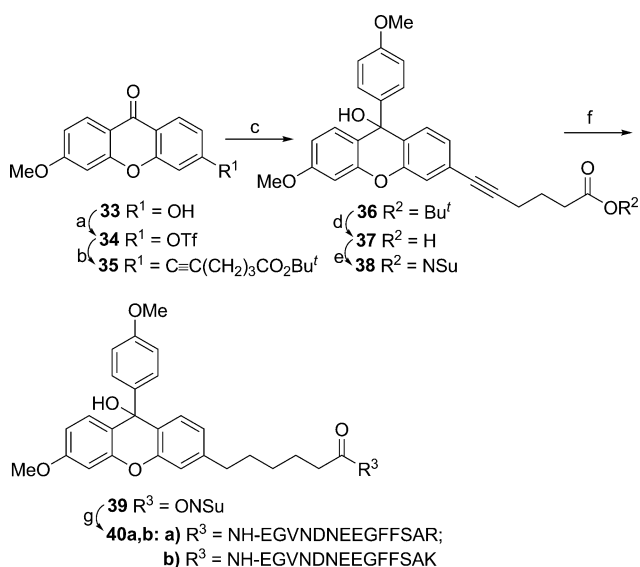
#	R ¹	Ar	R ³
32a	Me	4-C ₆ H ₄ OMe	(CH ₂) ₃ CH ₃

Scheme 4 Introduction of alkyl linker into benzophenone and further conversion into triarylmethanol derivatives. *Reagents and conditions*: a) BBr₃, DCM, rt; b) EtI, K₂CO₃, acetone, rt; c) BnCl, MeONa, HMPA, 100 °C; d) HC≡C(CH₂)₃CO₂Bu', Pd(PPh₃)₄, CuI, Et₃N, DMF, rt; e) H₂, Pd/C, EtOAc, rt; f) [¹³C]EtOH, DEAD, Ph₃P, THF, rt; g) ArMgBr, THF, rt; h) CF₃CO₂H, DCM, rt; i) DSC, Et₃N, DCM, rt; j) HONSu-SO₃Na, DCC, DMF, rt; k) R³NH₂, THF, rt.

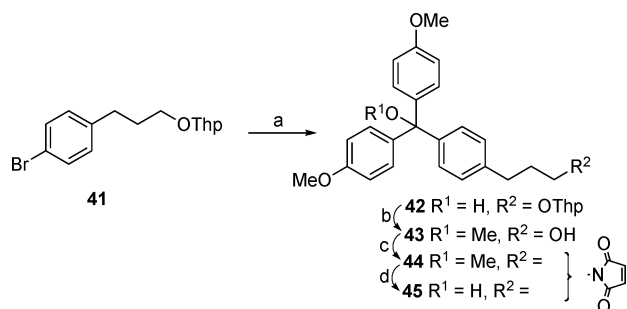
prepared several model compounds by combining several tags together (Chart 1). The first conjugate, **10g**, contained a trityl and a positively charged quaternary ammonium moiety. The MS analysis of this compound (Fig. 2) revealed only monocharged species: at 507 Da (loss of Cl) and at 490 Da (loss of both Cl and OH moieties). No molecular ion (542 Da), or its adducts, or any

double charged species were detected. Importantly, 525 Da peak ([MI - OH]⁺) was not detected either, neither with nor without (data not shown) matrix. This indicates that the trityl residue, upon losing the hydroxyl, exists apparently in a radical form.

Two trityl mass-tags were linked together by coupling NHS ester **15a** with diaminoethane. The resulting compound **17c** ionised well



Scheme 5 Introduction of alkyl linker into xanthone and further conversion into triarylmethanol derivatives. *Reagents and conditions*: a) Ti₂O, DIEA, DCM, rt; b) HC≡C(CH₂)₃CO₂Bu^t, Pd(PPh₃)₄, CuI, Et₃N, DMF, rt; c) ArMgBr, THF, rt; d) CF₃CO₂H, DCM, rt; e) DSC, Et₃N, DCM, rt; f) H₂, Pd/C, EtOAc, rt; g) R³NH₂, THF, rt.



Scheme 6 Preparation of dimethoxytritanol based maleimide reagent. *Reagents and conditions*: a) 1) BuLi, THF, -70 °C, 2) MeOC₆H₄COC₆H₄OMe, THF, -40 °C; b) CF₃CO₂H (cat.), MeOH; c) maleimide, DEAD, Ph₃P, toluene; d) CF₃CO₂H, H₂O.

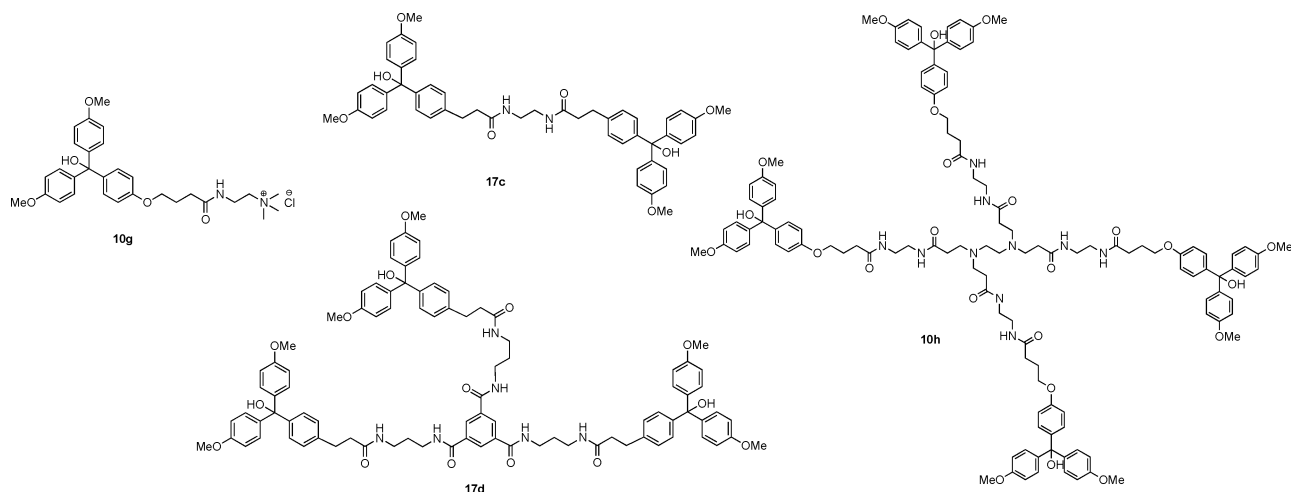
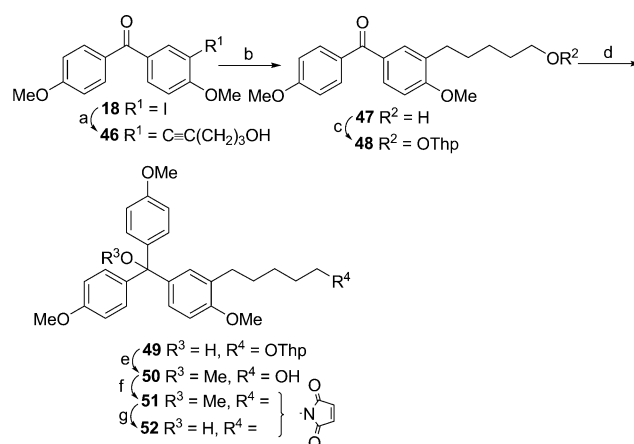


Chart 1 Structures of model compounds.



Scheme 7 Preparation of trimethoxytritanol based maleimide reagent. *Reagents and conditions*: a) HC≡C(CH₂)₃OH, Pd(PPh₃)₄, CuI, Et₃N, DMF, rt; b) H₂, Pd/C, EtOAc, rt; c) DHP, H⁺, DCM; d) MeOC₆H₄MgBr, THF, rt; e) CF₃CO₂H (cat.), MeOH; f) maleimide, DEAD, Ph₃P, toluene; g) CF₃CO₂H, H₂O.

without matrix, producing strong peaks of roughly equal intensity at 791 Da ([MI - OH]⁺) and 774 Da ([MI - 2OH]⁺) and no doubly charged species (Fig. 3). Since both hydroxyls are missing and the analyte desorbs as a monocharged ion, once again the suggestion regarding the state of the second trityl residue is that it is in a form of a radical.

A cluster of three tritanols linked together **17d** was prepared using trimesic acid as a core molecule. In all cases, only a singly charged peak was detected (Fig. 4A), although these di- and tri-tritanols were successively losing all the OH groups (1483 Da, [MI - OH]⁺; 1467 Da, [MI - 2OH]⁺; 1450 Da, [MI - 3OH]⁺; Fig. 4B).

In all of the above-described clusters, the trityl moieties are attached to the core through linkers flexible enough so that some conformations may allow for direct contact between trityl residues, decreasing the chance for several positive charges to co-exist in one molecule. To investigate this effect, as well as to further increase the number of trityls in a cluster, we have synthesised two more trityl blocks, one of which was flexible (**10h**) and another one not (**56**), linking four trityls through stiff arms (Scheme 8).

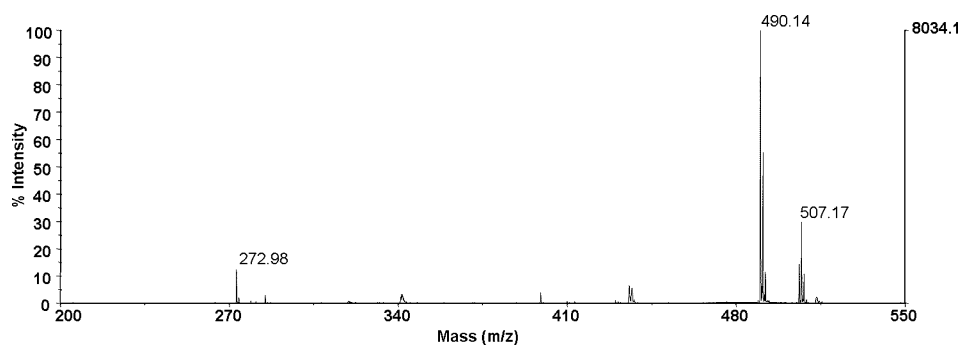


Fig. 2 MALDI-TOF analysis of compound **10g** (matrix: 0.5 mm³ of 20 mg/cm³ DHB was mixed with 0.5 mm³ of 1 mg/cm³ sample). No doubly charged species detected.

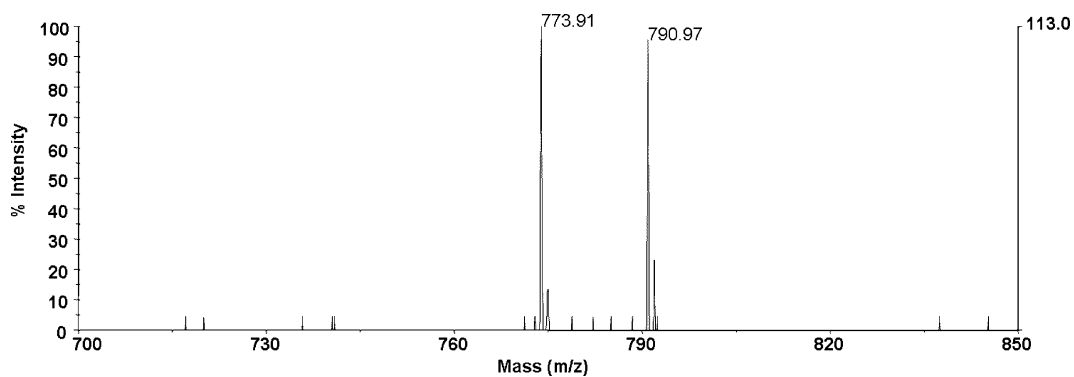


Fig. 3 LDI-TOF analysis of compound **17c**. No doubly charged species or molecular ion were detected.

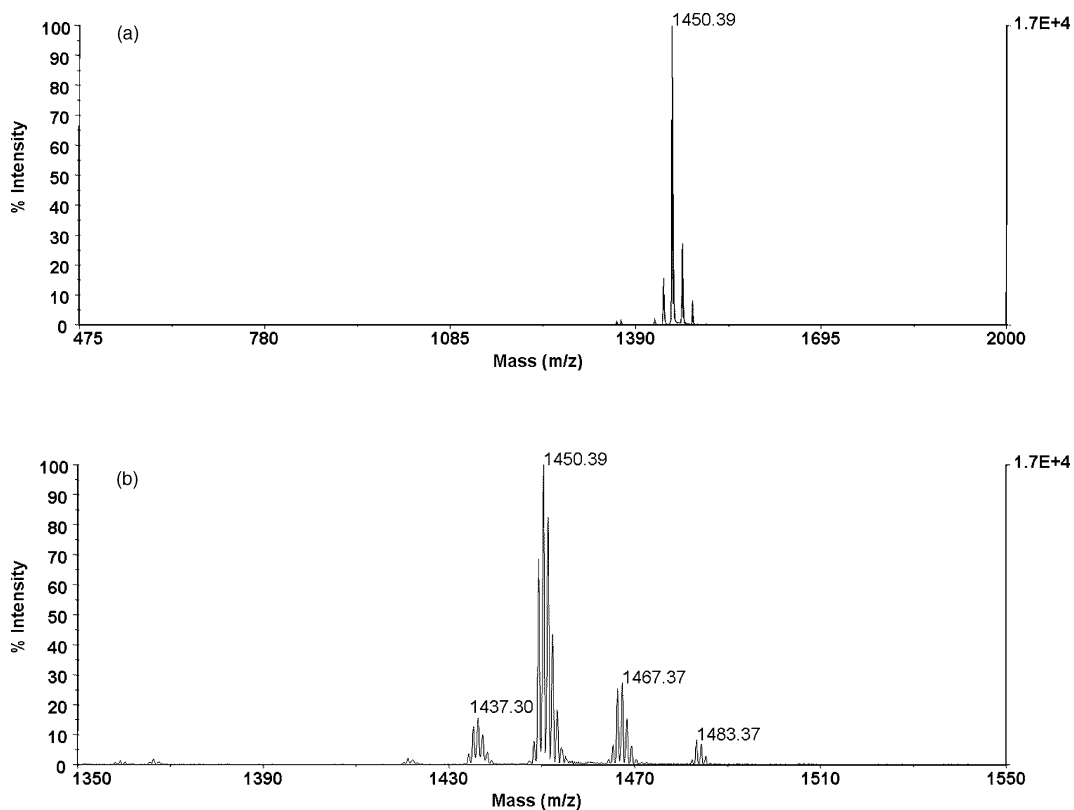
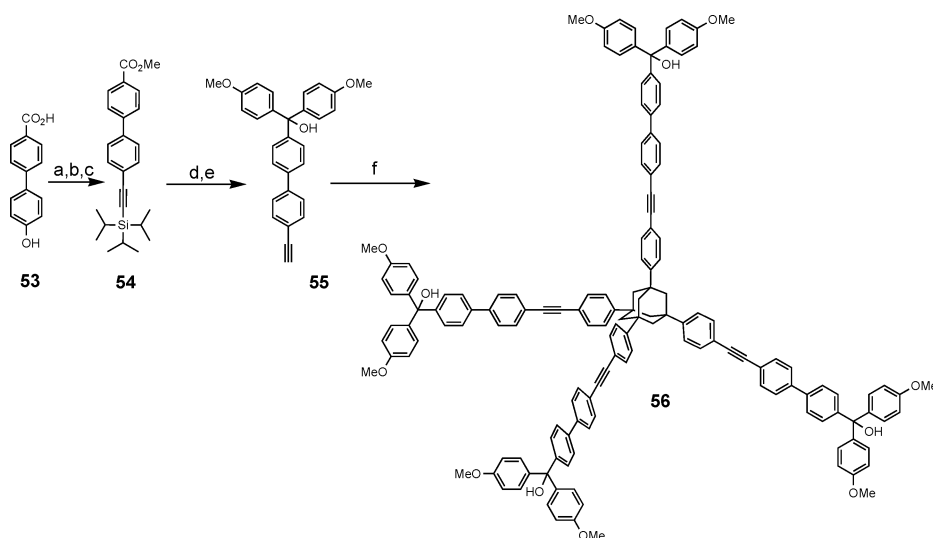


Fig. 4 MALDI-TOF analysis of compound **17d** (matrix: 0.5 mm³ of 20 mg/cm³ DHB was mixed with 0.5 mm³ of 1 mg/cm³ sample) No doubly/triply charged species or molecular ion were detected. The 1437 Da peak is [MI – 2OH – OMe]⁺.



Scheme 8 Synthesis of a stiff cluster bearing four trityls. *Reagents and conditions:* a) SOCl_2 , MeOH, rt; b) Ti_2O , Py, -20°C ; c) $\text{HC}\equiv\text{CSiPr}^t_3$, $\text{Pd}(\text{PPh}_3)_4$, CuI, Et_3N , DMF, rt; d) 4-MeOC₆H₄MgBr, THF, reflux 3 h; e) Bu_4NF , THF, rt; f) 1,3,5,7-tetra(4-iodophenyl)adamantane, $\text{Pd}(\text{PPh}_3)_4$, CuI, Et_3N , DMF, rt.

Once again, for both structures only a singly charged ion was detected, although the spectrum showed the consecutive loss of one, two, three and all four hydroxyls (2117 Da, $[\text{MI} - \text{OH}]^+$; 2100 Da, $[\text{MI} - 2\text{OH}]^+$; 2083 Da, $[\text{MI} - 3\text{OH}]^+$; 2065 Da, $[\text{MI} - 4\text{OH}]^+$; Fig. 5; and 2097 Da, $[\text{MI} - \text{OH}]^+$; 2080 Da, $[\text{MI} - 2\text{OH}]^+$; 2064 Da, $[\text{MI} - 3\text{OH}]^+$; and 2047 Da, $[\text{MI} - 4\text{OH}]^+$; Fig. 6).

This shows no difference in the charge state and the amount of hydroxyls lost between flexible and stiff or non flexible variants. Three different types of trityl moieties were used for these experiments, (8, 15a, 55), all with different pK_{R^+} values, thus ruling out the possible effect of the nature of trityls or the direct contacts of trityls within a molecule on its charge state.

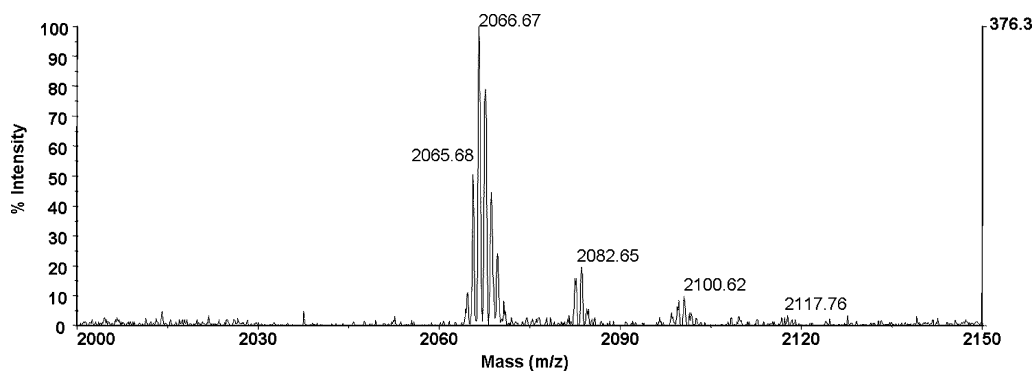


Fig. 5 MALDI-TOF analysis of compound 10h (matrix: 0.5 mm³ of 20 mg/cm³ DHB was mixed with 0.5 mm³ of 1 mg/cm³ sample). No doubly/triply/quadruply charged species or MI were detected.

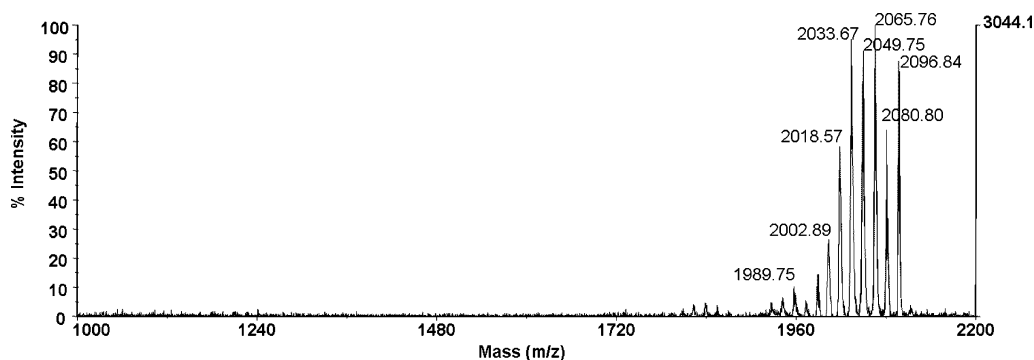


Fig. 6 MALDI-TOF analysis of compound 56 (matrix: 0.5 mm³ of 20 mg/cm³ DHB was mixed with 0.5 mm³ of 1 mg/cm³ sample). No doubly/triply/quadruply charged species or MI were detected. Peaks below 2050 Da correspond to species with cleaved methoxyl groups.

It is known from the literature that polytrityl cations exist and are stable.¹⁸ It is also known that, albeit to a lesser extent compared to ESI-MS, multiply charged cations can be detected by MALDI-TOF.¹⁹

On the other hand, it has been previously reported that peptides derivatised with two quaternary ammonium tags form only singly (and not doubly) charged ions in MALDI-TOF,^{9a} a finding earlier reported for model “double-quads”.²⁰ As multiply charged species are a nuisance that complicates the interpretation of the MS data, we were relieved to find out that our tags should not cause this problem, and moved on to test them with model peptides.

MS studies of peptide conjugates

We tested some of the trityl mass-tags for their ability to enhance the ionisation and desorption properties of peptides in MALDI-TOF. Typically, a bottom-up proteomics approach consists of digesting a set of proteins with trypsin, that yields peptides C-terminated with Lys or Arg, which are subsequently analysed by MS. These two types are known to ionise differently in conditions of MALDI-TOF: Arg-terminated peptides are detected on average 6 times better.²¹ But even within each of the two fractions the intensities differ, so that only some of peptides produced from a protein by an enzymatic digest are detectable by a MALDI-TOF technique, despite the fact that the peptides are present in equimolar amounts. In other words, there is a need to improve the ionisation ability of all peptides, if not making the intensities more uniform.

We have therefore investigated the MS enhancement by synthesising two sets of model peptides using a solid-phase approach. Glu-Fib peptide (EGVNDNEEGFFSAR), and its C-end Lys-

terminated analog (EGVNDNEEGFFSAK) were synthesised using Fmoc chemistry.²² The Fmoc groups were removed, and the Arg and Lys resin batches were then split into fractions and treated with NHS-activated trityl tags (2–5 cm³ of 0.05 M solutions in pyridine) for 15–20 minutes. The solid-bound conjugates were then cleaved, deprotected and purified to about 90% purity by ion-exchange HPLC using standard protocols, yielding 4–10 mg of each conjugate as well as the nonlabelled control.²² The structures prepared are shown in Table 1.

For each of two series (Arg and Lys), equimolar mixtures were prepared and analysed by MALDI-TOF (0.2 mm³ of 2.5 pmol/mm³ of each peptide conjugate was mixed with 0.2 mm³ of 10 mg/cm³ of α -cyano-4-hydroxycinnamic acid and spotted onto a MALDI target for analysis). For the Arg series (Fig. 7), all derivatives had more intense peaks (**17e**, 1928 Da; **10i**, 1971 Da; **40a**, 1983 Da; **28i**, 1998 Da) compared to the non-labelled **57** (1571 Da), with two derivatives (**17e** and **10i**) substantially improving the detection.

Results of the MALDI-TOF comparison of the Lys series are shown on Fig. 8. The spectrum shows that two trityl derivatives, **10j** (1942 Da) and **40b** (1955 Da) is detected better than the control **58** (1542 Da).

A more detailed analysis of **17e** (Glu-Fib labelled with the acid **14a**) revealed an interesting deisotoping pattern (Fig. 9). While the non-labelled **57** upon deisotoping produces a typical single peak (data not shown), the **17e** conjugate yields a “doublet”, with 1 Da mass difference. We believe that the doublet proves the presence of two different forms of **17e**. As our experiments with model compound **10g** and tritanol clusters **17c**, **17d**, **10h** and **56** suggest, tritanols can lose the hydroxyl and still not carry a charge, staying in a radical form instead. The charge for ionising the analyte

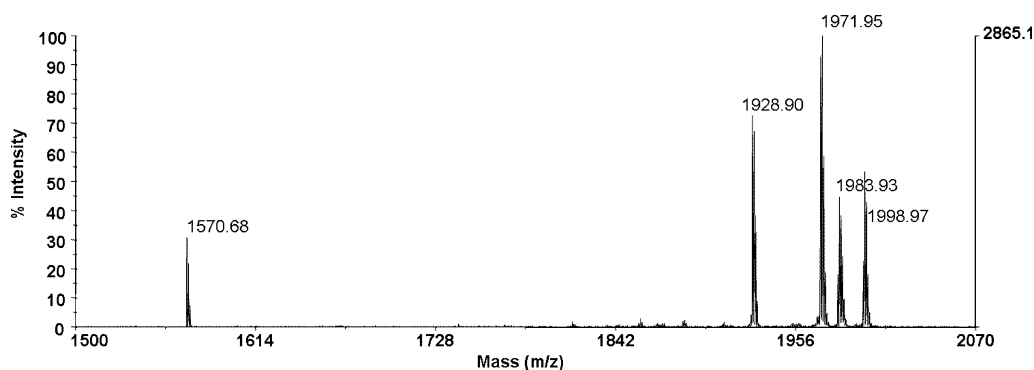


Fig. 7 Comparative MALDI-TOF analysis of an equimolar mixture of Glu-Fib **57** and its trityl-labelled derivatives **17e**, **10i**, **40a**, and **28i**.

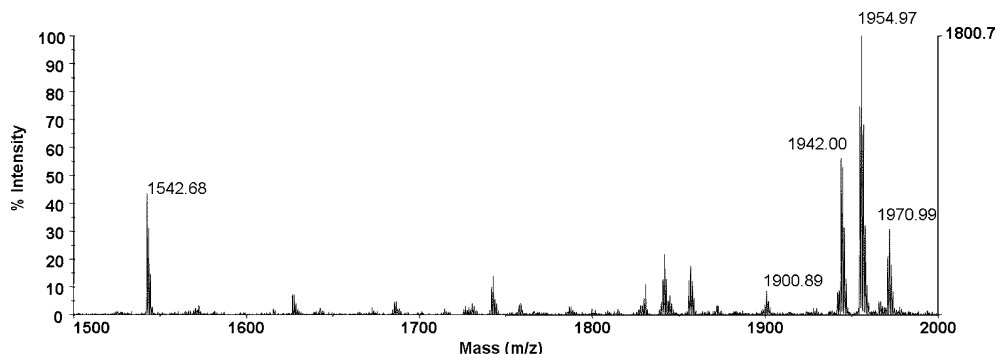


Fig. 8 Comparative MALDI-TOF analysis of an equimolar mixture of C-Lys-Glu-Fib **58** and its trityl-labelled derivatives **17f**, **10j**, **40b**, and **28j**.

Table 1 Peptide conjugates prepared using a solid phase method.²² The pK_{a+} values of the trityl MTs used were -1.5 (**17**), 6 (**40**) and 1.3 (**28**); the pK_{a+} value for **10** is not defined due to a more complex nature of cationisation of this compound

R		Peptide conjugate	
H			57
			17e
			10i
			40a
			28i
			58
			17e
			10j
			40b
			28j

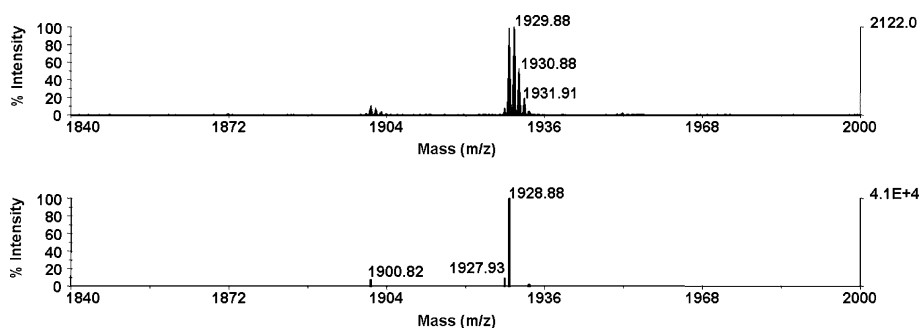


Fig. 9 MALDI-TOF analysis of **17e** (top), and deisotoping of the same spectrum (bottom).

should thus come from elsewhere – and in this case it comes from a protonated guanidyl residue of Arg; **17e** would therefore have a mass of $(MI - OH + H^+)$ Da. But this process could be in equilibrium with the trityl residue gaining the charge. Because double charging does not happen in these conditions (*vide supra*), this conjugate would therefore not be protonated and thus have a mass of $(MI - OH)$ Da. These two species, with a mass difference of 1 Da, are present simultaneously – as the two peaks with 1Da difference indicate. This is not the case for Lys terminated peptides, which have a lower basicity compared to Arg so that the charge would reside exclusively on the trityl.

Interestingly, the presence of this “doublet” in the Arg series was only observed for **17e**. The fact that **10i**, **40a** and **28i** are not detected in the $[MI - OH + H]^+$ form but only in the $[MI - OH]^+$ form (deisotoping data not shown) suggests that due to the higher pK_{R^+} values of their trityl MTs compared to **17e** all species would have the charge residing on the trityl. Thus, the attachment of trityl acid **14a** to a peptide, although it did not always improve its detection, allows one to distinguish easily arginine-containing peptides.²³

Finally, we have tested the efficiency of peptide labelling with activated forms of our trityl MTs in homogenous conditions. Labelling of GluFib peptide in aqueous acetonitrile with activated ester **16a** (See Experimental section) did not proceed to completion, as is obvious from Fig. 10. A solid-phase labelling method, which produces conjugates not contaminated with the starting peptides, will be reported elsewhere.

In some cases, double labelling was also detected (data not shown), involving both Lys and N-terminal amines. Non-complete conversion would require subsequent separation of non-labelled peptides from the reaction mix.

To test the labelling efficiency of maleimido-activated trityl MTs, we labelled a peptide, Laminin (CDPGYIGSR), with maleimido trityl **52** in acetonitrile (Fig. 11A). The efficiency of this reaction was comparable to that for **15a** (cf. Fig. 10). A substantial improvement was achieved when pyridine was used instead of acetonitrile (Fig. 11B), indicating that pyridine might be a better solvent in bioconjugation reactions involving trityl MTs (see Experimental section). Once again, a solid-phase labelling would provide a necessary clean-up for the conjugation reactions (see Scheme 1; $GR^2 =$ solid phase); solid-phase trityl MT reagents will be reported elsewhere.

Experimental

Instrumentation

500 MHz 1H and 125.7 MHz ^{13}C NMR spectra were recorded on a Bruker DRX-500 spectrometer and referenced to DMSO- d_6 (2.50 ppm and 39.5 ppm).²⁴ 1H NMR coupling constants are reported in Hz and refer to apparent multiplicities. 1H - ^{13}C gradient-selected HMQC and HMBC spectra were obtained by using $2048 (t_2) \times 256 (t_1)$ complex point data sets, zero filled to $2048 (F_2) \times 1024 (F_1)$ points. The spectral widths were 13 ppm and 200 ppm for 1H and ^{13}C dimensions, respectively. HMBC spectra were measured with 50 ms delay for evolution of long-range couplings. (MA)LDI-TOF mass spectra were obtained using a Voyager Elite Biospectrometry Research Station (PerSeptive Biosystems) in a positive ion mode. ESI-TOF HRMS spectra in positive ion mode were obtained using Micromass LCT reflection TOF mass spectrometer. Analytical thin-layer chromatography was performed on the Kieselgel 60 F₂₅₄ precoated aluminium plates

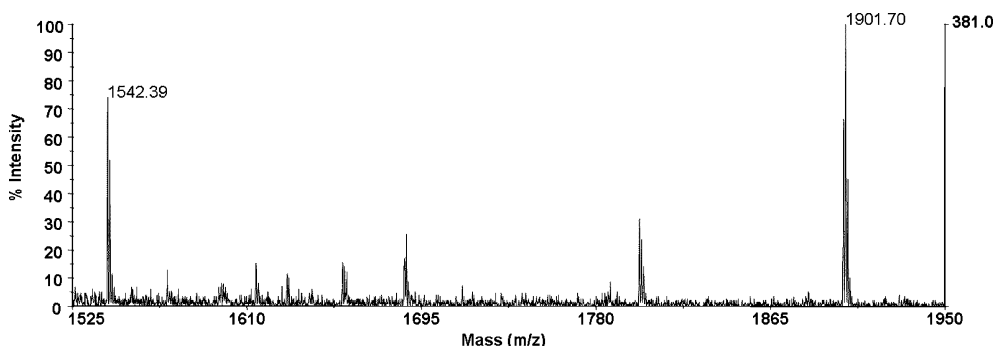


Fig. 10 Labelling of C-Lys-Glu-Fib **58** (1542 Da) with NHS-ester **15a**. 1901 Da is a monolabelled conjugate without the hydroxyl.

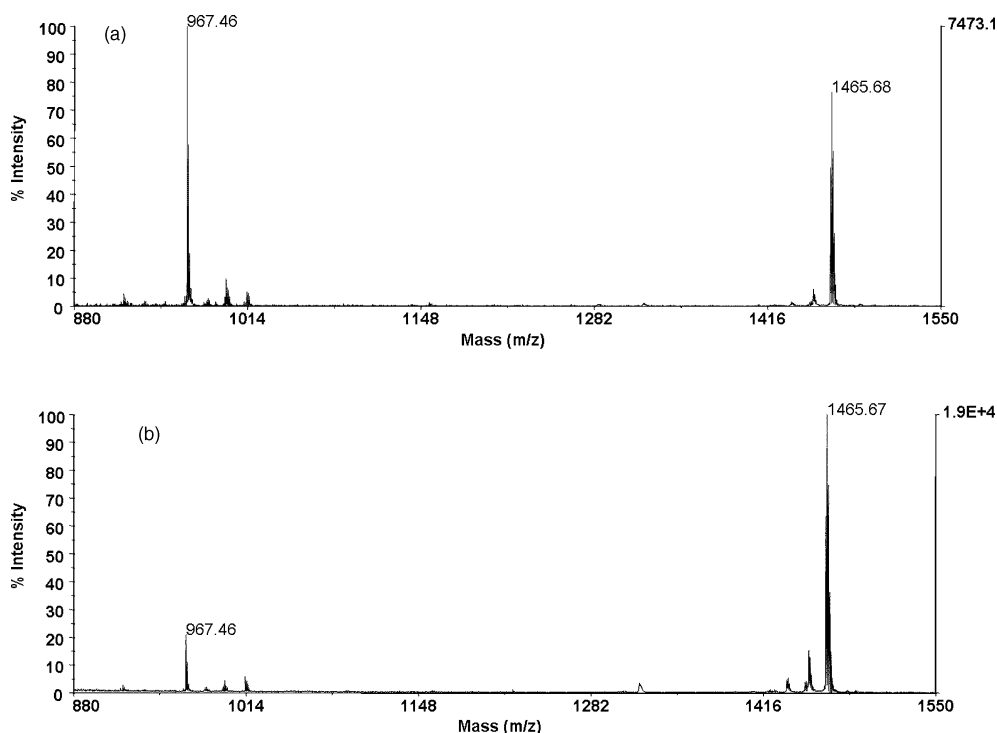


Fig. 11 Labelling of laminin (CDPGYIGSR) with maleimido-activated trityl **52** in acetonitrile (A) and in pyridine (B). Laminin, MW 967 Da; conjugate, MW 1465 Da (MI – OH).

(Merck), spots were visualised under UV light (254 nm). Column chromatography was performed on silica gel (Merck Kieselgel 60 0.040–0.063 mm).

Reagents and solvents

Reagents obtained from commercial suppliers were used as received. Solvents were mainly HPLC grade and used without further purification unless otherwise noted. DCM was always used freshly distilled over CaH₂. THF was distilled over powdered LiAlH₄ and stored over 4 Å molecular sieves under nitrogen. DMF was freshly distilled under reduced pressure.

Syntheses of activated trityl derivatives

3-[5-(*tert*-Butyloxycarbonyl)pent-1-ynyl]-4,4'-dimethoxybenzophenone (22a). To a solution of 3-iodo-4,4'-dimethoxybenzophenone (4.50 g, 12.2 mmol) and *tert*-butyl 5-hexynoate (2.10 g, 12.5 mmol) in DMF (50 cm³) Pd(PPh₃)₄ (1.40 g, 1.22 mmol), CuI (465 mg, 2.44 mmol), Et₃N (2.55 cm³, 18.3 mmol) were subsequently added. The mixture was stirred overnight under argon, then diluted with water (200 cm³) and extracted with EtOAc (200 cm³). The organic layer was washed with water (4 × 200 cm³), 0.1 M (NH₄)₂EDTA (4 × 200 cm³), dried over Na₂SO₄ and evaporated. The residue was purified by column chromatography in gradient of EtOAc in toluene (0 to 5%). Yield 3.43 g (69%), viscous yellowish oil. *R*_f 0.36 (10% EtOAc in PhMe (v/v)). ESI-TOF HRMS: *m/z* = 409.2027 [M + H]⁺, calc. for [C₂₅H₂₉O₅]⁺ 409.2010. ¹H NMR (DMSO-*d*₆): δ_H = 7.72–7.67 (m, 3H, H-6,2',6'); 7.64 (d, 1H, ⁴*J* = 2.1 Hz, H-2); 7.17 (d, 1H, *J* = 8.7 Hz, H-5); 7.08 (d, 2H, *J* = 8.7 Hz, H-3',5'); 3.91 (s, 3H, OCH₃); 3.86 (s, 3H, OCH₃); 2.47 (t, 2H, *J* = 7.0 Hz, ≡CCH₂); 2.37 (t, 2H, *J* =

7.4 Hz, COCH₂); 1.75 (m, 2H, CH₂CH₂CH₂); 1.39 (s, 9H, CCH₃). ¹³C NMR (DMSO-*d*₆): δ_C = 192.64 (ArCOAr), 171.86 (OCO), 162.74 (C4'), 162.60 (C4), 134.36 (C2), 131.86 (2C, C2',6'), 131.48 (C6), 129.93 (C1), 129.74 (C1'), 113.87 (2C, C3',5'), 112.23 (C3), 110.98 (C5), 94.42 (ArC≡C), 79.65 (C(CH₃)₃), 76.65 (ArC≡), 56.12 (4-OCH₃), 55.56 (4'-OCH₃), 33.67 (COCH₂), 27.79 (3C, C(CH₃)₃), 23.77 (CH₂CH₂CH₂), 18.30 (≡CCH₂).

3-[5-(*tert*-Butyloxycarbonyl)pentyl]-4,4'-dimethoxybenzophenone (23a). To a solution of 3-[5-(*tert*-butyloxycarbonyl)pent-1-ynyl]-4,4'-dimethoxybenzophenone (2.027 g, 4.96 mmol) in EtOAc (40 cm³) 10% Pd/C (100 mg) was added and the mixture was hydrogenated at 150 Torr for 24 h, filtered, diluted with EtOAc (150 cm³), washed with 5% NaHCO₃ (100 cm³) and 0.1 M (NH₄)₂EDTA (100 cm³), dried over Na₂SO₄, evaporated, and the residue was purified by column chromatography (0→10% EtOAc in toluene). Yield 1.493 g (73%), colourless oil. *R*_f 0.45 (10% EtOAc in PhMe (v/v)). ESI-TOF HRMS: *m/z* = 413.2304 [M + H]⁺, calc. for [C₂₅H₃₃O₅]⁺ 413.2323. ¹H NMR (DMSO-*d*₆): δ_H = 7.69 (d, 2H, *J* = 8.7 Hz, H-2',6'); 7.58 (dd, 1H, *J* = 8.4 Hz, ⁴*J* = 2.2 Hz, H-6); 7.53 (d, 1H, ⁴*J* = 2.2 Hz, H-2); 7.07 (m, 3H, H-5,3',5'); 3.88 (s, 3H, OCH₃); 3.86 (s, 3H, OCH₃); 2.59 (t, 2H, *J* = 7.4 Hz, ArCH₂); 2.16 (t, 2H, *J* = 7.3 Hz, COCH₂); 1.57–1.47 (m, 4H, COCH₂CH₂CH₂CH₂); 1.36 (s, 9H, CCH₃); 1.28 (m, 2H, COCH₂CH₂CH₂). ¹³C NMR (DMSO-*d*₆): δ_C = 193.38 (ArCOAr), 172.28 (OCO), 162.51 (C4'), 160.51 (C4), 131.81 (2C, C2',6'), 131.08 (C2), 130.23 (C3), 130.09 (C6), 129.93 (C1), 129.67 (C1'), 113.77 (2C, C3',5'), 110.17 (C5), 79.33 (C(CH₃)₃), 55.65 (4-OCH₃), 55.33 (4'-OCH₃), 34.73 (COCH₂), 29.31 (ArCH₂), 28.75 (ArCH₂CH₂), 28.17 (ArCH₂CH₂CH₂), 27.79 (3C, C(CH₃)₃), 24.48 (COCH₂CH₂).

3-[5-(*tert*-Butyloxycarbonyl)pentyl]-4,4',4''-trimethoxytritanol (24a). To a stirred solution 3-[5-(*tert*-butyloxycarbonyl)pentyl]-4,4'-dimethoxybenzophenone (412 mg; 1.0 mmol) in dry THF (10 cm³) 1 M 4-methoxyphenylmagnesium bromide (1.2 cm³, 1.2 mmol) was added in one portion under argon, and the mixture was kept at ambient temperature overnight (monitoring by TLC in EtOAc–toluene 1:3). The reaction was diluted with water (50 cm³) and saturated aq. NH₄Cl (20 cm³), and then extracted with EtOAc (2 × 100 cm³). The organic phase was dried over Na₂SO₄, evaporated, and chromatographed on aluminium oxide in 0→10% EtOAc in toluene with 0.5% of Et₃N to give the desired compound as a colourless oil. Yield 274 mg (53%). *R*_f 0.43 (10% EtOAc in PhMe (v/v)). ESI-TOF HRMS: *m/z* = 503.2811 [M–OH]⁺, calc. for [C₃₂H₃₉O₅]⁺ 503.2792. ¹H NMR (DMSO-*d*₆): δ_H = 7.06 (m, 4H, H-2',6',2'',6''); 6.97 (d, 1H, ⁴*J* = 1.9 Hz, H-2); 6.88–6.79 (m, 6H, H-5,6, 3',5',3'',5''); 6.02 (s, 1H, OH); 3.74 (s, 3H, OCH₃); 3.72 (s, 6H, OCH₃); 2.45 (t, 2H, *J* = 7.5 Hz, ArCH₂); 2.12 (t, 2H, *J* = 7.3 Hz, COCH₂); 1.44 (m, 4H, COCH₂CH₂CH₂CH₂); 1.37 (s, 9H, CCH₃); 1.20 (m, 2H, COCH₂CH₂CH₂). ¹³C NMR (DMSO-*d*₆): δ_C = 172.27 (CO), 157.76 (2C, C4',4''), 155.61 (C4), 140.68 (2C, C1',1''), 140.08 (C1), 129.25 (C3), 128.89 (4C, C2',6',2'',6''), 128.64 (C2), 126.36 (C6), 112.68 (4C, C3',5',3'',5''), 109.35 (C5), 79.67 (Ar₃COH), 79.34 (C(CH₃)₃), 55.31 (4-OCH₃), 55.02 (2C, 4'-OCH₃, 4''-OCH₃), 34.74 (COCH₂), 29.62 (ArCH₂), 28.99 (ArCH₂CH₂), 28.14 (ArCH₂CH₂CH₂), 27.79 (3C, C(CH₃)₃), 24.45 (COCH₂CH₂).

3-[5-(Succinimid-1-yloxycarbonyl)pentyl]-4,4',4''-trimethoxytritanol (26a). To a stirred solution of 3-[5-(*tert*-butyloxycarbonyl)pentyl]-4,4',4''-trimethoxytritanol (274 mg; 0.53 mmol) in dry DCM (2 cm³) trifluoroacetic acid (2 cm³) was added in one portion and the mixture was stirred at ambient temperature for 3 h, then evaporated, and co-evaporated with DCM (4 × 50 cm³) to give free acid. An analytical sample of the acid **25a** was purified by column chromatography on silica gel (20→30% acetone in toluene). *R*_f 0.30 (30% Me₂CO in PhMe (v/v)). ¹H NMR (DMSO-*d*₆): δ_H = 11.92 (br.s, 1H, CO₂H); 7.07 (d, 4H, *J* = 8.7 Hz, H-2',6',2'',6''); 6.98 (d, 1H, ⁴*J* = 1.9 Hz, H-2); 6.88 (dd, 1H, *J* = 8.4 Hz, ⁴*J* = 1.9 Hz, H-6); 6.85–6.79 (m, 5H, H-5, 3',5',3'',5''); 6.02 (br.s, 1H, OH); 3.75 (s, 3H, OCH₃); 3.72 (s, 6H, OCH₃); 2.45 (t, 2H, *J* = 7.8 Hz, ArCH₂); 2.15 (t, 2H, *J* = 7.3 Hz, COCH₂); 1.45 (m, 4H, COCH₂CH₂CH₂CH₂); 1.22 (m, 2H, COCH₂CH₂CH₂). ¹³C NMR (DMSO-*d*₆): δ_C = 174.47 (CO₂H), 157.76 (2C, C4',4''), 155.63 (C4), 140.68 (2C, C1',1''), 140.10 (C1), 129.29 (C3), 128.89 (4C, C2',6',2'',6''), 128.68 (C2), 126.37 (C6), 112.70 (4C, C3',5',3'',5''), 109.38 (C5), 79.68 (Ar₃COH), 55.32 (4-OCH₃), 55.03 (2C, 4'-OCH₃, 4''-OCH₃), 33.69 (CH₂CO₂H), 29.66 (ArCH₂), 29.11, 28.36, 24.37 (ArCH₂CH₂CH₂CH₂). The product was dissolved in DCM (15 cm³), and triethylamine (0.60 cm³, 4.3 mmol) and *N,N*-disuccinimidyl carbonate (556 mg, 2.17 mmol) were added and the mixture was stirred overnight, then evaporated, dissolved in EtOAc (50 cm³), washed with 5% NaHCO₃ (50 cm³) and water (50 cm³), dried over Na₂SO₄, evaporated, and the residue was purified by column chromatography (15→30% EtOAc in toluene). Yield 277 mg (93%), pink amorphous solid. *R*_f 0.64 (30% Me₂CO in PhMe (v/v)). ESI-TOF HRMS: *m/z* = 544.2388 [M–OH]⁺, calc. for [C₃₂H₃₄NO₇]⁺ 544.2330. ¹H NMR (DMSO-*d*₆): δ_H = 7.71 (d, 1H, *J* = 8.9 Hz, H-5); 7.07 (d, 4H, *J* =

8.9 Hz, H-2',6',2'',6''); 6.99 (d, 1H, ⁴*J* = 2.1 Hz, H-2); 6.89–6.78 (m, 5H, H-6,3',5',3'',5''); 6.02 (s, 1H, OH); 3.89 (s, 3H), 3.86 (s, 6H) (OCH₃); 2.80 (s, 4H, COCH₂CH₂CO); 2.61 (t, 2H, *J* = 7.3 Hz), 2.46 (t, 2H, *J* = 7.6 Hz) (ArCH₂CH₂CH₂CH₂CH₂); 1.54 (m, 2H), 1.44 (m, 2H), 1.32 (m, 2H) (ArCH₂CH₂CH₂). ¹³C NMR (DMSO-*d*₆): δ_C = 171.22 (CO₂N), 170.37 (2C, COCH₂CH₂CO), 157.75 (2C, C4',4''), 155.66 (C4), 140.64 (2C, C1',1''), 140.12 (C1), 129.28 (C3), 128.87 (4C, C2',6',2'',6''), 128.66 (C2), 126.35 (C6), 112.68 (4C, C3',5',3'',5''), 109.36 (C5), 79.65 (Ar₃COH), 55.30 (4-OCH₃), 55.01 (2C, 4'-OCH₃, 4''-OCH₃), 33.67 (CH₂CO₂H), 29.64 (ArCH₂), 29.10, 28.35, 24.39 (ArCH₂CH₂CH₂CH₂), 22.48 (2C, COCH₂CH₂CO).

3-(5-Hydroxypent-1-ynyl)-4,4'-dimethoxybenzophenone (46). was prepared similar to **22a** from 3-iodo-4,4'-dimethoxybenzophenone (3.16 g, 8.58 mmol), 4-pentynol-1 (1.20 cm³, 12.9 mmol) PdCl₂ (152 mg, 0.86 mmol), PPh₃ (450 mg, 1.72 mmol), CuI (82 mg, 0.43 mmol), Et₃N (2.4 cm³, 17.2 mmol) in DMF (30 cm³) and purified by chromatography on silica gel in gradient of EtOAc in toluene (0 to 50%). Yield 2.55 g (91%), viscous yellowish oil. *R*_f 0.47 (EtOAc). ESI-TOF HRMS: *m/z* = 325.1427 [M + H]⁺, calc. for [C₂₀H₂₁O₄]⁺ 325.1434. ¹H NMR (DMSO-*d*₆): δ_H = 7.72–7.66 (m, 3H, H-6,2',6'); 7.63 (d, 1H, ⁴*J* = 2.1 Hz, H-2); 7.17 (d, 1H, *J* = 8.9 Hz, H-5); 7.09 (d, 2H, *J* = 8.6 Hz, H-3',5'); 4.50 (t, 1H, *J* = 5.1 Hz, OH); 3.91 (s, 3H), 3.86 (s, 3H) (OCH₃); 3.52 (m, 2H, CH₂OH); 2.48 (t, 2H, *J* = 7.0 Hz, ≡CCH₂); 1.68 (m, 2H, CH₂CH₂CH₂). ¹³C NMR (DMSO-*d*₆): δ_C = 192.67 (ArCOAr), 162.72 (C4'), 162.58 (C4), 134.34 (C2), 131.85 (2C, C2',6'), 131.45 (C6), 129.91 (C1), 129.74 (C1'), 113.86 (2C, C3',5'), 112.21 (C3), 110.97 (C5), 94.38 (ArC≡C), 76.63 (ArC≡), 60.35 (CH₂OH), 56.10 (4-OCH₃), 55.56 (4'-OCH₃), 30.18 (CH₂CH₂CH₂), 14.95 (≡CCH₂).

3-(5-Hydroxypentyl)-4,4'-dimethoxybenzophenone (47). A solution of 3-(5-hydroxypent-1-ynyl)-4,4'-dimethoxybenzophenone (4.15 g, 12.8 mmol) in EtOAc (50 cm³) was hydrogenated as above in the presence of 10% Pd/C (300 mg). The product was isolated by chromatography on silica gel (0 to 50% gradient of EtOAc in toluene); white solid (3.32 g, 79%). *R*_f 0.76 (EtOAc), mp 101–102° (EtOH). ESI-TOF HRMS: *m/z* = 329.1698 [M + H]⁺, calc. for [C₂₀H₂₅O₄]⁺ 329.1747. ¹H NMR (DMSO-*d*₆): δ_H = 7.70 (d, 2H, *J* = 8.8 Hz, H-2',6'); 7.58 (dd, 1H, *J* = 8.7 Hz, ⁴*J* = 2.1 Hz, H-6); 7.53 (d, 1H, ⁴*J* = 2.1 Hz, H-2); 7.10–7.05 (m, 3H, H-5,3',5'); 4.31 (t, 1H, *J* = 5.0 Hz, OH); 3.88 (s, 3H), 3.86 (s, 3H) (OCH₃); 3.38 (m, 2H, CH₂OH); 2.60 (t, 2H, *J* = 7.6 Hz, ArCH₂); 1.54 (m, 2H), 1.44 (m, 2H), 1.32 (m, 2H), CH₂CH₂CH₂CH₂CH₂. ¹³C NMR (DMSO-*d*₆): δ_C = 193.43 (ArCOAr), 162.52 (C4'), 160.53 (C4), 131.84 (2C, C2',6'), 131.08 (C2), 130.26 (C3), 130.24 (C6), 129.93 (C1), 129.66 (C1'), 113.76 (2C, C3',5'), 110.17 (C5), 60.73 (CH₂OH), 55.77 (4-OCH₃), 55.54 (4'-OCH₃), 32.41 (CH₂CH₂OH), 29.54 (ArCH₂), 29.09 (ArCH₂CH₂), 25.44 (ArCH₂CH₂CH₂).

3-[5-(Tetrahydropyran-2-yloxy)pentyl]-4,4'-dimethoxybenzophenone (48). To a solution of 3-(5-hydroxypentyl)-4,4'-dimethoxybenzophenone (542 mg, 1.65 mmol) in DCM (10 cm³) DHP (0.17 cm³, 1.82 mmol) and TsOH·H₂O (16 mg, 0.08 mmol) were added and the mixture was stirred for 1.5 h at ambient temperature (TLC control in 10% EtOAc in toluene). Solid NaHCO₃ (0.5 g) was added and after 5 min the mixture was filtered, evaporated, and the residue was chromatographed on silica gel (3% EtOAc

and 1% Et₃N in toluene) to give the desired compound as a colourless oil (441 mg, 65%). *R*_f 0.33 (10% EtOAc in PhMe (v/v) + 1% Et₃N). ESI-TOF HRMS: *m/z* = 413.2341 [M + H]⁺, calc. for [C₂₅H₃₃O₃]⁺ 413.2323. ¹H NMR (DMSO-*d*₆): δ_H = 7.78 (d, 2H, *J* = 8.5 Hz, H-2',6'); 7.63 (m, 2H, ⁴*J*_{2,6} = 1.8 Hz, H-2,6); 6.95 (d, 2H, *J* = 8.5 Hz, ArH, H-3',5'); 6.87 (d, 1H, *J* = 8.2 Hz, H-5); 4.56 (m, 1H, OCHO); 3.92–3.82 (m, 7H, CH₃, OCHH); 3.73 (m, 1H), 3.48 (m, 3H), 3.38 (m, 3H) (OCHH); 2.64 (t, 2H, *J* = 7.6 Hz, ArCH₂); 1.88–1.38 (m, 12H, OCH₂CH₂CH₂CH₂). ¹³C NMR (DMSO-*d*₆): δ_C = 193.40 (ArCOAr), 162.51 (C4'), 160.55 (C4), 131.84 (2C, C2',6'), 131.09 (C2), 130.23 (C3), 130.22 (C6), 129.92 (C1), 129.65 (C1'), 113.74 (2C, C3',5'), 110.16 (C5), 97.98 (OCHO), 65.69 (CH₂OThp), 61.18 (Thp), 55.75 (4-OCH₃), 55.52 (4'-OCH₃), 32.40 (CH₂CH₂OH), 30.35 (Thp), 29.54 (ArCH₂), 29.07 (ArCH₂CH₂), 25.42 (ArCH₂CH₂CH₂), 25.10, 19.20 (Thp).

3-[5-(Tetrahydropyran-2-yloxy)pentyl]-4,4',4''-trimethoxytritanol (49). To a solution of 3-[5-(Tetrahydropyran-2-yloxy)pentyl]-4,4'-dimethoxybenzophenone (363 mg, 0.88 mmol) in THF (5 cm³) 1.6 M 4-methoxyphenylmagnesium bromide in THF (0.75 cm³, 1.2 mmol) was added in one portion and the mixture was kept overnight at ambient temperature. The mixture was quenched by addition of water (1 cm³) and evaporated. The residue was dissolved in EtOAc (30 cm³), washed with 5% NaHCO₃ (2 × 10 cm³), dried over Na₂SO₄ and evaporated. The desired compound was isolated by column chromatography on silica gel (5% EtOAc + 1% Et₃N in toluene) as a colourless oil (304 mg, 66%). *R*_f 0.49 (20% EtOAc in PhMe (v/v) + 1% Et₃N). ESI-TOF HRMS: *m/z* = 503.2771 [M–OH]⁺, calc. for [C₃₂H₃₀O₅]⁺ 503.2792. ¹H NMR (DMSO-*d*₆): δ_H = 7.07 (d, 4H, *J* = 8.7 Hz, H-2',6',2'',6''); 6.99 (d, 1H, ⁴*J* = 2.3 Hz, H-2); 6.87 (dd, 1H, *J* = 8.4 Hz, ⁴*J* = 2.3 Hz, H-6); 6.82 (m, 5H, H-5,3',5',3'',5''); 6.02 (s, 1H, OH); 4.50 (m, 1H, OCHO); 3.74 (s, 3H), 3.72 (s, 6H) (OCH₃); 3.69 (m, 1H), 3.57 (m, 1H), 3.40 (m, 1H), 3.27 (m, 1H) (2 OCH₂); 2.47 (t, 2H, *J* = 7.4 Hz, ArCH₂), 1.73–1.63 (m, 1H), 1.61–1.53 (m, 1H), 1.51–1.37 (m, 8H), 1.27 (m, 2H) (2 OCH₂CH₂CH₂CH₂). ¹³C NMR (DMSO-*d*₆): δ_C = 157.76 (2C, C4',4''), 155.63 (C4), 140.70 (2C, C1',1''), 140.08 (C1), 129.29 (C3), 128.89 (4C, C2',6',2'',6''), 128.72 (C2), 126.37 (C6), 112.67 (4C, C3',5',3'',5''), 109.33 (C5), 97.94 (OCHO), 79.68 (Ar₃COH), 66.59 (CH₂OThp), 61.26 (Thp), 55.29 (4-OCH₃), 55.01 (2C, 4'-OCH₃, 4''-OCH₃), 30.37 (Thp), 29.78 (ArCH₂), 29.18, 29.08, 25.54 (ArCH₂CH₂CH₂CH₂), 25.11, 19.23 (Thp).

3-(5-Hydroxypentyl)-4,4',4''-trimethoxytrityl methyl ether (50). To a stirred solution of 3-[5-(tetrahydropyran-2-yloxy)pentyl]-4,4',4''-trimethoxytritanol (304 mg; 0.58 mmol) in MeOH (20 cm³) TsOH·H₂O (10 mg) was added, and the mixture was kept at ambient temperature overnight (monitoring by TLC in EtOAc–toluene 1:9). Solid K₂CO₃ (300 mg) was added and the mixture was evaporated and chromatographed on silica gel in 20% EtOAc + 1% Et₃N in toluene. Yield 254 mg (97%). *R*_f 0.23 (20% EtOAc in PhMe (v/v) + 1% Et₃N). ESI-TOF HRMS: *m/z* = 419.2201 [M–OMe]⁺, calc. for [C₂₇H₃₁O₄]⁺ 419.2217. ¹H NMR (DMSO-*d*₆): δ_H = 7.24 (d, 4H, *J* = 8.5 Hz, H-2',6',2'',6''); 7.10 (dd, 1H, *J* = 8.5 Hz, ⁴*J* = 2.1 Hz, H-6); 7.07 (d, 1H, ⁴*J* = 2.1 Hz, H-2); 6.88 (m, 5H, H-5,3',5',3'',5''); 4.30 (t, 1H, *J* = 5.0 Hz, OH); 3.76 (s, 3H), 3.74 (s, 3H) (ArOCH₃); 3.36 (m, 2H, CH₂OH), 2.92 (s, 3H, Ar₃COCH₃); 2.49 (t, 2H, *J* = 7.4 Hz, ArCH₂); 1.49–1.36 (m, 4H, ArCH₂CH₂CH₂CH₂); 1.25 (m, 2H, ArCH₂CH₂CH₂). ¹³C NMR

(DMSO-*d*₆): δ_C = 157.90 (2C, C4',4''), 155.76 (C4), 136.42 (2C, C1',1''), 135.58 (C1), 129.76 (C3), 129.42 (4C, C2',6',2'',6''), 128.93 (C2), 126.99 (C6), 113.08 (4C, C3',5',3'',5''), 109.83 (C5), 85.61 (Ar₃COCH₃), 60.78 (CH₂OH), 55.31 (4-OCH₃), 55.03 (2C, 4'-OCH₃, 4''-OCH₃), 51.24 (Ar₃COCH₃), 32.43, 29.82, 29.30, 25.28 (ArCH₂CH₂CH₂CH₂).

3-[5-(1-Maleimido)pentyl]-4,4',4''-trimethoxytritanol (52). To a stirred solution of 3-(5-hydroxypentyl)-4,4',4''-trimethoxytrityl methyl ether (642 mg; 1.42 mmol) in toluene (10 cm³) maleimide (152 mg, 1.57 mmol), PPh₃ (410 mg, 1.57 mmol) were added followed by DEAD (0.26 cm³, 1.64 mmol). The mixture was kept at ambient temperature for 24 h (monitoring by TLC in 20% EtOAc in PhMe (v/v) + 1% Et₃N; *R*_f 0.52 (intermediate **51**), and evaporated. The crude compound **51** was dissolved in toluene (10 cm³). Trifluoroacetic acid (0.22 cm³, 2.84 mmol) was added and after 10 minutes the mixture was quenched by saturated aqueous NaHCO₃. Organic layer was separated and washed with aqueous NaHCO₃ (10 cm³), water (10 cm³) and brine (10 cm³), dried over Na₂SO₄ and evaporated. The residue was chromatographed on silica gel in 10→18% EtOAc in toluene + 1% Et₃N. Yield 384 mg (51%). *R*_f 0.42 (20% EtOAc in PhMe (v/v) + 1% Et₃N). ESI-TOF HRMS: *m/z* = 498.2290 [M–OH]⁺, calc. for [C₃₁H₃₂NO₅]⁺ 498.2275. ¹H NMR (DMSO-*d*₆): δ_H = 7.07 (d, 4H, *J* = 8.7 Hz, H-2',6',2'',6''); 6.98 (s, 2H, COCH=CHCO); 6.96 (d, 1H, ⁴*J*_{2,6} = 2.1 Hz, H-2); 6.86 (dd, 1H, ⁴*J*_{2,6} = 2.1 Hz, *J* = 8.5 Hz, H-6); 6.85–6.79 (m, 5H, H-5,3',5',3'',5''); 6.01 (s, 1H, OH); 3.74 (s, 3H), 3.72 (s, 6H) (OCH₃); 3.34 (t, 2H, *J* = 7.4 Hz, NCH₂); 2.43 (t, 2H, *J* = 7.4 Hz, ArCH₂); 1.45 (m, 4H, ArCH₂CH₂CH₂CH₂); 1.19 (m, 2H, ArCH₂CH₂CH₂). ¹³C NMR (DMSO-*d*₆): δ_C = 171.07 (2C, CO), 157.76 (2C, C4',4''), 155.63 (C4), 140.69 (2C, C1',1''), 140.10 (C1), 134.45 (2C, COCH=CHCO), 129.20 (C3), 128.89 (4C, C2',6',2'',6''), 128.60 (C2), 126.40 (C6), 112.70 (4C, C3',5',3'',5''), 109.35 (C5), 79.67 (COH), 55.31 (4-OCH₃), 55.02 (2C, 4'-OCH₃, 4''-OCH₃), 37.06 (CH₂N), 29.56 (ArCH₂), 28.89, 27.77, 25.89 (ArCH₂CH₂CH₂CH₂).

Labelling procedures

NHS-ester Labelling. The labelling reaction was carried out with freshly made 0.02 M stock solution of the water-soluble labelling reagent **16a** dissolved in 80% 200mM NaHCO₃/20% acetonitrile. 4 mm³ of this stock solution was added to 25 mm³ (200 pmol) of Glu-Fib-K peptide in 80% 200 mM NaHCO₃/20% acetonitrile and left for 1h at room temperature. 2 mm³ of the reaction mixture was purified by micro-extraction and before MALDI-TOF MS analysis. The sample was diluted with 0.1% trifluoroacetic acid (TFA) (15 mm³) and applied to C₁₈ Zip Tip (prepared by washing with 99.9% MeCN/0.1% TFA (3 × 10 mm³) followed by 0.1% TFA (3 × 10 mm³)). The sample was applied to C₁₈ Zip Tip and passed through 5–6 times. The Zip Tip was washed with 0.1% TFA (5 × 10 mm³), and peptides were eluted with 50% MeCN/0.1% TFA (3 mm³). 0.2 mm³ of the eluted sample was mixed with α-cyano-4-hydroxycinnamic acid in 50% MeCN/0.1% TFA (10 mg/cm³; 0.2 mm³) and applied onto MALDI target plate.

Maleimido-activated trityl Labelling. The labelling reaction was carried out with freshly made 0.02 M stock solution of the maleimido labelling reagent **52** dissolved in acetonitrile. 4 mm³ of this stock solution was added to 25 mm³ (200 pmol) of Laminin

peptide in either acetonitrile or pyridine. This was left shaking for 30 minutes at room temperature. 1 mm³ of the reaction mixture was purified by micro-extraction and before MALDI-TOF MS analysis. The sample was diluted with 0.1% trifluoroacetic acid (TFA) (12 mm³) and purified on C₁₈ Zip Tip and analysed as above.

Conclusions

We have designed functionalised trityl MTs for MS applications. This work has produced a large number of different trityl structures of which a few have been tested as possible mass tags. The MS studies of peptide conjugates reported here are initial results for four selected trityl derivatives. These MTs were used for labelling of trypsin digests and yielded more detectable peptides (higher MASCOT score) than corresponding non-labelled mixtures; trityl MT labelling also improved some aspects of ESI-MS studies. Although some of these MTs have shown an enhancement of signal for the conjugated peptides, we have also seen some derivatives that, surprisingly, suppress the signal (e.g. Fig. 8, **17f** and **28j**). We are continuing to optimise the design of functionalised trityl MTs for MS applications (mainly in the proteomic field), and more development must be carried out to further investigate this trend with a wider range of peptides. We are also in the process of developing more efficient methods for labelling including a solid-phase method.

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