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Trityl Tags for Encoding in Combinatorial Synthesis

Mikhail S. Shchepinov,* Rod Chalk and Edwin M. Southern

Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

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Abstract—New tags and an encoding strategy for combinatorial synthesis are described. Combinatorial libraries of short oligonucleotides attached to TentaGel beads were synthesised by a split-and-mix strategy using 5′-DMTr or 5′-Fmoc-protected nucleoside phosphoramidites. Trityl moieties with different masses were used to tag the nature and position of monomer units (bases) coupled at each step in the synthesis. Beads with a specific oligonucleotide were selected by hybridisation from combinatorial libraries. Tags orthogonal to the added nucleotides were produced by coupling amines of different molecular masses to an activated carboxyl group(s) on the trityl moiety. The tags may be released from the support by an acidic treatment or laser irradiation and then analysed by (MA)LDI-TOF. These properties make trityl-based tags promising for encoding in strategies not involving strong acids, such as oligonucleotide and peptide synthesis and small molecule combinatorial libraries.

Introduction

The combinatorial approach to simultaneous synthesis of large numbers of chemical compounds on solid supports, first used for screening in electronic materials,[1] has been an important development in biological and medicinal chemistry,[2–4] allowing the use of rapid screening to speed up lead identification and optimisation. Two methods predominate: spatially addressable (or parallel) arrays, in which synthesis steps are performed simultaneously on sets of visibly separated starting materials;[5,6] and bead libraries, consisting of mixtures of microscopic resin beads each of which carries a few pmol or μmol of a single compound, usually synthesised using the 'split-and-mix' method.[7,8] Although resin libraries are efficient in their use of chemicals and quickly screened, their application is limited if the compound on a selected 'hit' bead cannot be readily identified.

One way around this is a deconvolution strategy, but these are wasteful of both chemicals and time, and parallel synthesis cannot always accommodate large numbers of compounds.[9] The alternative is to attach one or more tags to the bead, which can be cleaved and identified even at very low concentration, and which will encode the synthesis steps that bead has undergone. The problem of encoding during 'one-bead–one-compound' combinatorial synthesis has been addressed by several groups and recently reviewed.[10] A successful encoding tag must survive the synthesis and assay, be cleaved specifically and orthogonally to the tethered compound, and be readily identifiable in pico- or femtomol quantities. To obtain orthogonal chemistry oligonucleotides (which may be amplified by polymerase chain reaction (PCR)[11]), secondary amines[12] and haloaryls[13] (binary coding) have been used as encoding tags, with varying degrees of success. Identification has been carried out by most spectroscopic and chromatographic methods, including HPLC,[12] GC,[14,15] mass spectrometry,[16–24] fluorescence,[25] IR[26,27] and NMR spectroscopy.[28] Libraries of limited size have been encoded by using physical separation, either 'tea-bags'[29] or mini-capsules containing radio-frequency microchips, which can then be identified using an electronic reader.[30,31] Physical encoding by altering the size, shape, density and colour of the beaded support has also been investigated.[32] However, none of these methods has been particularly successful at encoding large libraries, such as those formed by a complete set of all possible oligonucleotides of a defined length. The sheer numbers involved point to the use of bead libraries for their synthesis and screening, but the limited sensitivity of gel-based sequencing methods rules out direct identification.

Triarylmethyl (trityl)-type cations are stabilised by the resonance effect of the phenyl rings, which makes their ethers acid labile, and they are consequently a useful family of protective groups, especially in nucleoside chemistry.[33–36] Trityl groups generating cations of different colours have been used to protect different nucleotides in oligonucleotide synthesis.[37] Modified trityl groups have been used, for example, to accelerate the formation of internucleotide bonds in the phosphotriester approach,[38,39] to reversibly label synthetic oligonucleotides with biotin etc.[40,41] to purify them by immobilising on to a solid support after synthesis,[42]

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* Corresponding author. Tel.: +44-1865-275-226; fax: +44-1865-275-283; e-mail: misha@bioch.ox.ac.uk

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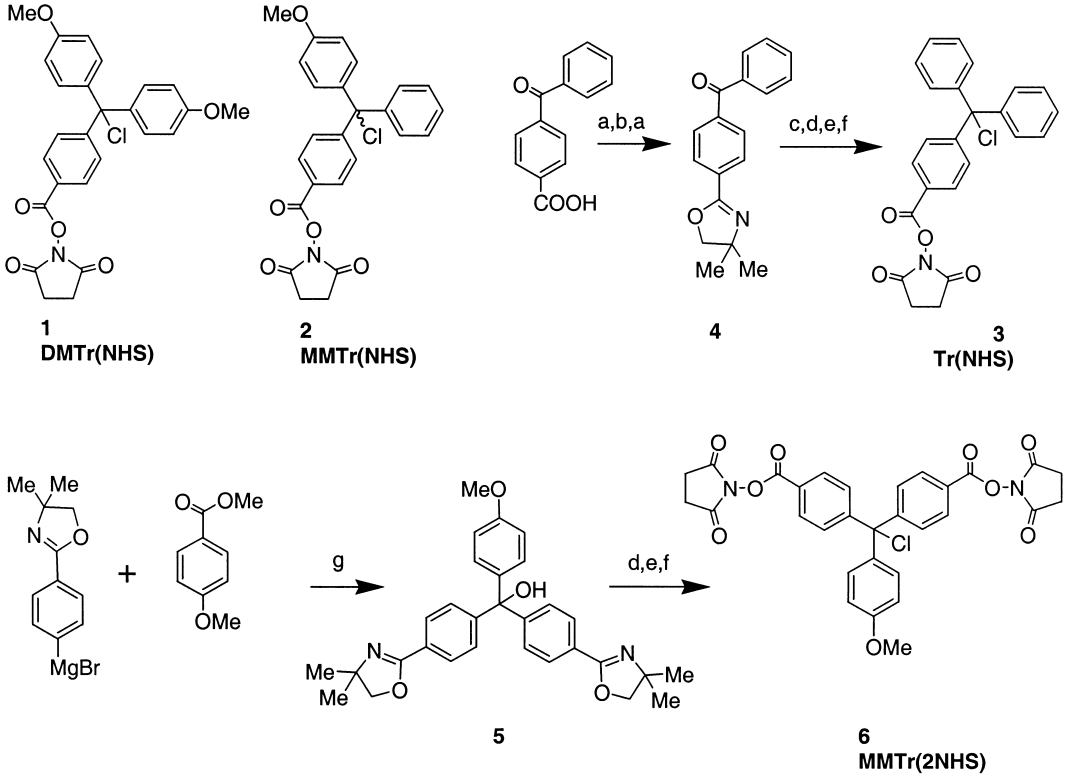


Scheme 1. (a) $SOCl_2$, reflux. (b) 2-Amino-2-methylpropan-1-ol, 2.5 equiv. (c) PhMgBr. (d) 80% AcOH, 48 h. (e) NHS, DCC. (f) AcCl, toluene, reflux. (g) Grignard synthesis. Alternatively, **3** may be synthesised from benzophenone and 4-bromophenyl oxazoline.

to quantify the amount of amino groups on a solid support,[43] and to controllably activate prodrug antibody conjugates.[44–46] A modified trityl group bearing a pyrenyl residue in place of one of the aryl groups has been used for more precise fluorescent detection (down to 10^{-10} M) of detritylation,[47] and a ^{14}C-labelled dimethoxytrityl (DMTr) group was used for more sensitive monitoring of coupling reactions on an aminated polypropylene support.[48] Apart from acidic treatment, some alternative ways of removing the triarylmethyl group have been explored, including treatment with anion-radicals[49,50] and $ZnBr_2$.[51] Importantly, for the tags described in this paper, it has also been reported that the trityl cation may form merely upon irradiation of the starting material by photochemical ionisation.[52]

The characteristic signal of the $DMTr^+$ cation (monoisotopic peak at 303.139 Da) is frequently present in mass-spectra of DMTr-containing compounds, suggesting that derivatives of trityl groups with different masses could serve as the unique mass-tags in combinatorial synthesis. We describe here a new method of encoding, which is based on the high desorption rate of triphenylmethyl cation-based tags in the conditions of laser desorption/ionisation TOF mass-spectrometry, which makes detection simpler than in previously described encoding systems.[13–16] The trityl groups can be released by acidic treatment and detected by laser desorption/ionisation TOF analysis with or without matrix. Alternatively, the cations can be generated directly by laser irradiation.

Results and Discussion

Synthesis of activated trityl blocks

A dimethoxytrityl group bearing an *N*-hydroxysuccinimide (NHS)-activated carboxyl function (**1**) was previously used for the reversible labelling of oligonucleotides and for other applications.[40–42,44–46] We used Köster's approach[40,41] to synthesise trityl groups that are more stable to acid (both with and without methoxy groups (Scheme 1)). To avoid the synthesis of the Grignard reagent from 2-(4-bromophenyl)-4,4-dimethyl-1,3-oxazoline, we took advantage of the commercially available Grignard reagents (Aldrich) and synthesised oxazolyl-protected 4-carboxybenzophenone (**4**) starting from 4-carboxybenzophenone. Following the Grignard reaction with phenylmagnesium bromide, subsequent steps were similar to those used for compounds **1** and **2**.[40,41]

Treating activated carboxyl group-containing trityl synthons with different amines generates a variety of tags with different masses. The masses of the majority of cheap commercially available primary amines, which would withstand the conditions of oligonucleotide synthesis and deprotection (thus excluding, for example, all aromatic amines unless phenoxyacetyl (PAC)- or similar 'fast' phosphoramidites, are used for the combinatorial synthesis) lie mainly in the range of 50–250 Da. For some applications it is desirable to have several hundred mass-tags available. The resolution of the tags in TOF mass-spectrometry was found to be satisfactory with $\geq$2 Da difference between the masses

Table 1. Registered masses (LDI-TOF conditions, no matrix) of four different trityl-based pro-tags **1**, **2**, **3** and **6** treated with 80 amines. Dashed line indicates no signal detected

#	MW of amines, Da	Chemical name	Tr(NHS) (3)	MMTr(NHS) (2)	DMTr(NHS) (1)	MMTr(NHS) (6)
1.	17.03	Ammonia	286.27	316.25	346.31	359.36
2.	31.06	Methylamine	300.33	330.32	360.43	387.45
3.	45.09	Ethylamine	314.38	344.35	374.45	415.54
4.	59.11	Propylamine	328.39	358.44	388.53	443.64
5.	73.14	Butylamine	342.47	372.46	402.51	471.71
6.	74.09	Glycinamide (xHCl)	–	372.44	403.60	474.55
7.	85.15	Cyclopentylamine	354.42	384.47	414.53	495.71
8.	87.17	Amylamine	356.43	386.60	416.52	499.73
9.	87.17	2-Amino-3-methylbutane	356.38	386.42	416.50	499.65
10.	89.14	2-Amino-1-methoxypropane	358.35	388.34	418.14	503.57
11.	89.14	4-Amino-1-butanol	358.40	388.39	418.41	503.65
12.	89.14	2-Amino-2-methyl-1-propanol	358.39	388.43	418.51	503.66
13.	97.12	Furfurylamine	366.43	396.42	426.54	519.64
14.	99.18	Cyclohexylamine	368.47	398.52	428.58	523.78
15.	101.19	Hexylamine	370.56	400.55	430.69	527.76
16.	103.17	5-Amino-1-pentanol	372.52	402.60	432.63	531.78
17.	103.19	Thiomorpholine	372.47	402.46	432.55	531.72
18.	105.14	2-(2-Aminoethoxy)-ethanol	374.45	404.53	434.56	535.66
19.	113.20	Cycoheptylamine	382.50	412.49	442.58	551.86
20.	114.19	1-(2-Aminoethyl)-pyrrolidine	383.43	413.45	443.59	553.68
21.	115.22	Heptylamine	384.49	414.48	444.55	555.81
22.	121.18	Phenethylamine	390.52	420.48	450.48	567.63
23.	122.17	2-(2-Aminoethyl)-pyridine	391.62	421.54	451.63	569.84
24.	125.18	1-(3-Aminopropyl)-imidazole	394.24	424.58	454.73	576.34
25.	127.23	Cyclooctylamine	396.62	426.61	456.77	580.00
26.	128.18	α-Amino-ϵ-caprolactam	397.56	427.54	457.65	581.91
27.	128.22	2-(2-Aminoethyl)-1-methylpyrrolidine	397.64	427.67	457.71	–
28.	128.22	1-(2-Aminoethyl)-piperidine	–	427.62	457.69	581.91
29.	129.25	Octylamine	398.64	428.57	458.70	583.92
30.	130.19	4-(2-Aminoethyl)-morpholine	399.56	429.49	459.60	585.94
31.	130.23	N,N-Diethyl-1,3-propanediamine	399.53	429.55	459.66	585.85
32.	135.17	3-Phenylpropylamine	404.54	434.51	464.66	595.72
33.	135.21	1-(4-Methoxyphenyl)-ethylamine	404.59	434.55	464.65	595.89
34.	137.18	4-Methoxybenzylamine	406.57	436.55	466.63	599.85
35.	137.18	2-Phenoxyethylamine	406.61	436.55	466.56	599.91
36.	139.17	4-Fluoro-α-methyl-benzylamine	408.62	438.58	468.63	603.91
37.	142.20	1-(3-Aminopropyl)-2-pyrrolidinone	411.63	411.67	471.77	610.02
38.	143.14	3,5-Difluorobenzylamine	412.56	442.55	472.67	611.85
39.	143.27	Nonylamine	412.67	442.65	472.74	612.10
40.	144.22	4-(3-Aminopropyl)-morpholine	413.57	443.52	473.58	–
41.	147.22	1,2,3,4-Tetrahydro-1-naphthylamine	416.55	446.49	476.59	619.82
42.	149.06	2,2,3,3,3-Pentafluoropropylamine	418.50	448.46	478.58	623.78
43.	149.24	1-Methyl-3-phenylpropylamine	418.65	448.61	478.58	623.95
44.	149.24	4-Phenylbutylamine	418.64	448.61	478.73	623.95
45.	151.21	Norephedrin	420.62	450.63	480.77	627.98
46.	151.21	2-(4-Methoxyphenyl)-ethylamine	420.63	450.66	480.71	627.95
47.	151.25	1-Adamantylamine	420.69	450.62	480.75	628.06
48.	155.29	4-tert-Butylcyclohexylamine	424.73	454.68	480.75	636.06
49.	155.29	Menthylamine	424.68	454.63	484.77	635.82
50.	156.27	1-(3-Aminopropyl)-2-pipecoline	–	455.63	485.79	–
51.	157.22	1-Naphtalenemethylamine	426.52	456.49	486.55	639.69
52.	157.30	Decylamine	426.68	456.64	486.70	640.02
53.	158.29	2-Amino-5-diethylaminopentane	427.75	457.67	487.81	642.12
54.	160.22	Tryptamine	428.67	459.64	489.72	645.97
55.	162.24	1-Phenylpiperazine	431.69	461.70	491.75	649.99
56.	167.21	2,6-Dimethoxybenzylamine	436.63	466.60	496.65	659.94
57.	171.33	Undecylamine	440.85	470.76	500.88	668.21
58.	175.15	4-(Trifluoromethyl)-1-benzylamine	444.65	475.66	504.52	676.03
59.	176.26	1-Benzyl-3-aminopyrrolidine	445.72	–	505.74	677.93
60.	180.23	1-(4-Fluorophenyl)-piperazine	449.60	477.64	509.71	685.94
61.	181.25	Dicyclohexylamine	–	–	–	–
62.	183.25	Aminodiphenylmethane	452.60	482.57	512.63	691.80
63.	185.36	Dodecylamine	454.80	484.79	514.86	696.16
64.	190.29	4-Amino-1-benzylpiperidene	459.73	489.69	519.83	706.00
65.	191.27	2-Benzyloxycyclopentylamine	460.80	490.68	520.54	708.02
66.	193.15	3-Fluoro-5-(trifluoromethyl)-benzylamine	462.75	492.60	522.64	711.88
67.	197.28	1,2-Diphenylethylamine	466.75	496.68	526.83	719.96
68.	199.38	Tridecylamine	468.90	498.85	528.93	724.30
69.	200.26	4-(2-Aminoethyl)benzenesulfonamide	469.59	499.59	529.68	725.81
70.	206.75	1-(2-Ethoxyphenyl)-piperazine (xHCl)	477.71	502.68	535.99	–
71.	207.28	Amino-2,2-dimethyl-4-phenyl-1,3-dioxane	476.53	506.49	536.44	752.22

Table 1 (*continued*)

#	MW of amines, Da	Chemical name	Tr(NHS) (3)	MMTr(NHS) (2)	DMTr(NHS) (1)	MMTr(NHS) (6)
72.	213.41	Tetradecylamine	482.81	512.79	542.81	752.30
73.	221.31	9-(Methylaminomethyl)-anthracene	490.77	520.83	–	–
74.	227.44	Pentadecylamine	496.88	526.90	557.03	780.21
75.	231.30	1-Pyrenemethylamine	509.83	540.93	571.08	808.36
76.	241.46	Hexadecylamine	512.74	542.71	572.78	812.17
77.	243.15	3,5-Bis(trifluoromethyl)-benzylamine	–	–	–	860.46
78.	269.50	Octadecylamine	538.99	568.92	599.15	864.52
79.	297.57	Didecylamine	566.97	596.99	627.02	920.55
80.	339.61	Hexetidine	609.00	638.99	–	–

of tags. Therefore, the above range of amines can only yield a limited number of tags. Secondary amines are not useful because usually there are primary amines with the same masses, and generally secondary amines are less reactive owing to steric hindrance (cf. entries ##14 and 61, Table 1). Amines bearing other reactive groups, for example, ##11, 12 and 18 (Table 1), cannot be used as tags in the conditions of oligonucleotide synthesis unless they are introduced at the very last step or an additional capping step is employed. Finally, some amines are unstable to the deprotection conditions (e.g. #13). To increase the number of mass-tags using readily available amines, we synthesised an additional trityl-based reagent containing two NHS-activated carboxyl groups (**6**), which allows attachment of two amines to the same trityl moiety thus extending the series of mass-tags into the higher mass range (**9**, Scheme 1).

To evaluate these modified trityl blocks as precursors for mass-tags, compounds **1** and **2** were used to synthesise 5′-protected thymidine.[40–42] 0.1 M solutions of these nucleosides, and also of compounds **3** and **6** in the OH-form (tritanols), in THF were reacted with 0.5–1 M solutions of amines in THF or dioxane (5 equiv. of amine for mono-NHS-based compounds **1**–**3** and 10 equiv. for **6**), by mixing 200 μl of each of tritylated compounds with the corresponding amount of an amine solution and allowing them to react for 5 min. The reaction mixtures were then analysed by mass spectrometry without matrix, to prevent the formation of molecular ions, by applying 1 μl of these mixtures directly on to a sample target plate and allowing them to dry. Typical results are presented in Table 1. For all trityl derivatives, the compounds that gave the strongest signal were selected and analysed as a mixture by mixing all and applying 1 μl of the mixture to the target plate. Typical spectra are shown in Fig. 1(a)–(c). There is a certain decrease in the signal intensities when the tags are analysed as a mixture, which can partially be explained by the decreased concentration of individual tags in the mixture. But even in mixtures, the signal intensities are high enough to distinguish the tags unambiguously. For applications in which the tags can be removed prior to LDI-TOF analysis (Scheme 2), the use of matrix may further increase the signal intensities (Fig. 1(c)).

All the synthesised trityl tags (**1**–**3**, **6**) and standard DMTr, monomethoxytrityl (MMTr) and triphenylmethyl (trityl, Tr) groups were tested for acid-lability by treatment of the corresponding 5′-thymidylates with 1–3% TsOH, $HClO_4$ or 80% aqueous acetic acid and TLC-analysis of the products after quenching with sat. $NaHCO_3$ (data not shown). As expected, there was about one order of magnitude difference in stabilities between DMTr, MMTr and Tr. The corresponding carboxy-derivatives were about two to five times more stable, i.e. the stability was: DMTr< DMTr(NHS) (**1**)~MMTr<MMTr(NHS) (**2**)<MMTr(2NHS) (**6**)~Tr<Tr(NHS) (**3**). The MMTr(NHS) group (**2**) is about 50 times more stable than the DMTr group. There was no detectable difference between the acidic stabilities of NHS-activated trityl groups and their corresponding amide derivatives. Interestingly, the stability of the modified trityls correlated well with their relative signal intensities (Fig. 2). No difference was detected for the signal intensity for tritanols as compared to the corresponding trityl ethers when using laser ionisation instead of acidic treatment (data not shown), suggesting photocleavage by the laser irradiation[52] as a good alternative to acidic cleavage.

Encoding of combinatorial libraries

For the 300 μm Rapp beads used in our experiments, the average mass of a single bead is about 10 μg, which, given the capacity of the reactive hydroxyl groups on that polymer as 0.21 mmol/g, gives a loading of ~2 nmol per bead; more usually, for the supports used for combinatorial chemistry it is ~200 pmol/bead.[56] Assuming some loss of reactive groups due to incomplete synthesis, we would still have about 1 nmol of reactive groups per bead, a few percent of which would be tagged by the scheme used in this study (vide infra). Two-fold and ten-fold dilution experiments (data not shown) showed that the lower limit of LDI-TOF detection of trityl-based tags is around 10^{-13} M concentration level (per single tag). The diameter of the spot on the sample well, which is covered by the laser beam, is about 100–300 μm, which means that the actual amount of sample necessary for detection is in the fmol range. With about 5% of sites occupied by tags, one bead provides about 50 pmol of tags, which is more than enough for detection.

To be used as a tag in oligonucleotide synthesis, the trityl group should give clean high intensity signal in (MA)LDI-TOF analysis. It should also survive several steps of acidic treatment used to remove the 5′-DMTr group in oligonucleotide synthesis, that is, be orthogonal to the other protective groups involved. (The stability of the NHS-group in conditions of oligonucleotide synthesis has been proved elsewhere.[40,41]) We selected the MMTr(NHS) group (**2**) to meet both these requirements. Unlike DMTr(NHS) (**1**), it remains attached to a primary hydroxyl group after at

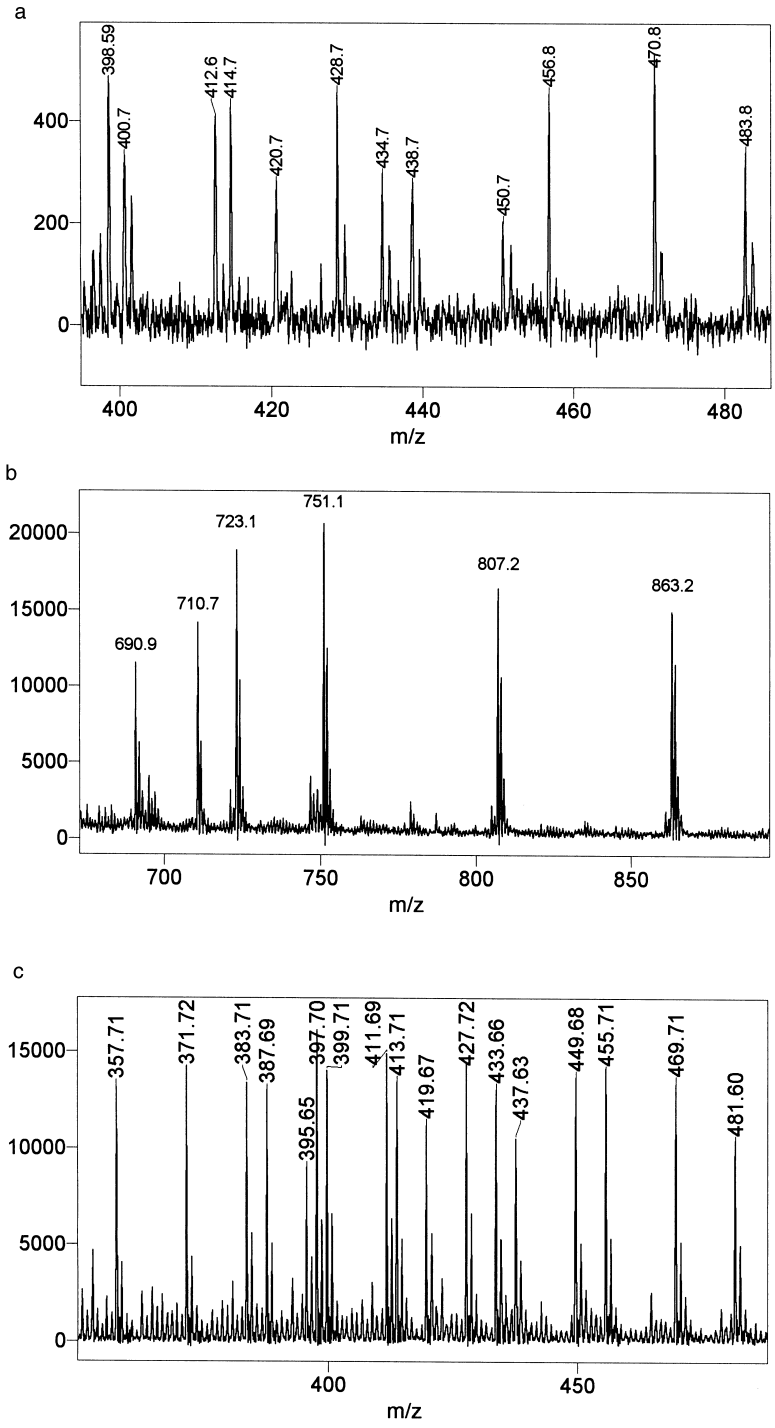
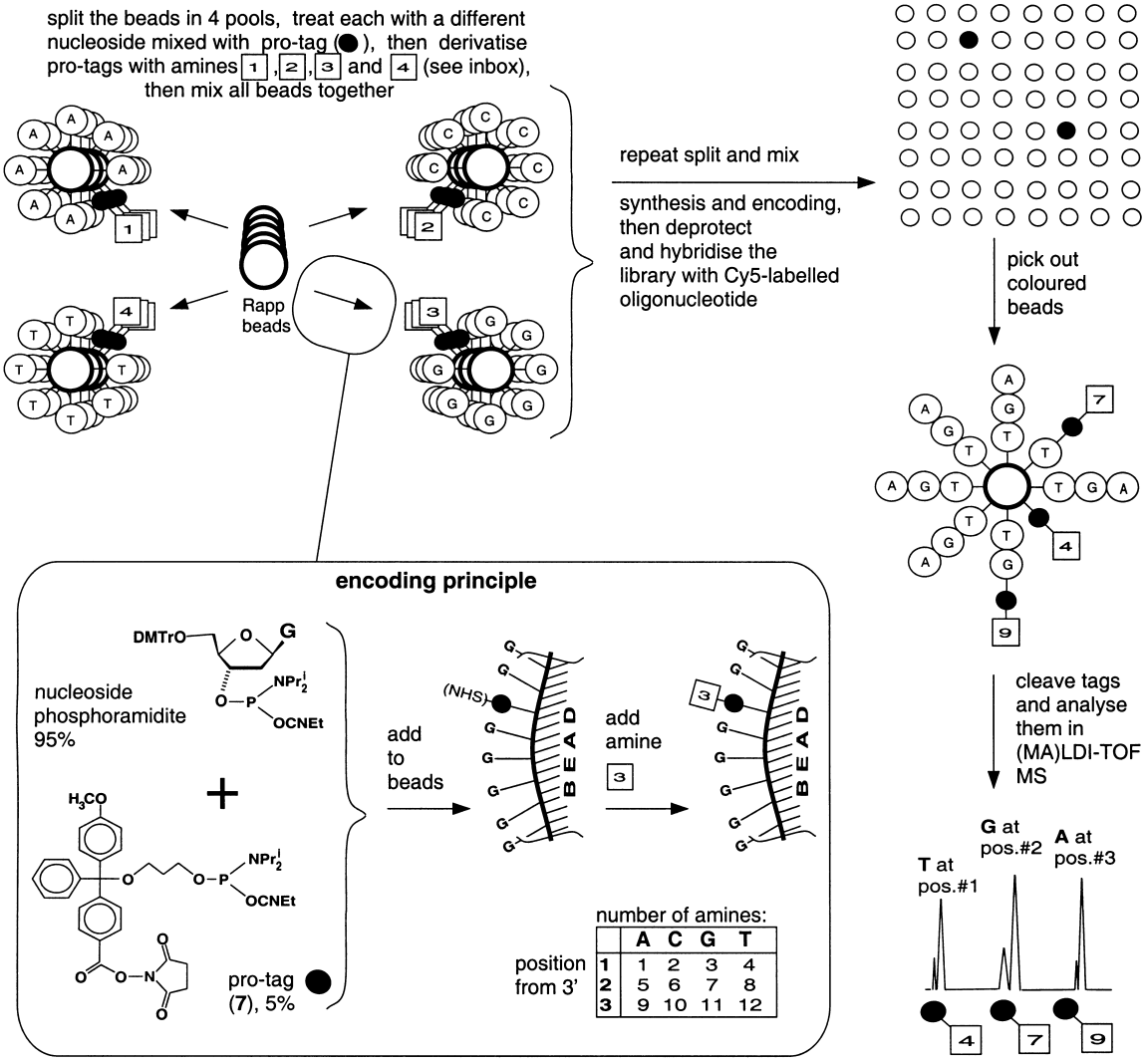


Figure 1. (a) Selection of mass-tags based on compound **2** (MMTr(NHS)) treated with amines ## 14, 15, 19, 21, 22, 29, 32, 36, 46, 52, 57 and 62. Analysed as a mixture of **8**, without matrix. (b) Selection of mass-tags based on compound **6** (MMTr(2NHS)) treated with amines ## 62, 66, 68, 72, 75 and 78. Analysed as a mixture of **9**, without matrix. (c) Selection of mass-tags based on compound **2** (MMTr(NHS)) treated with amines ## 4, 5, 7, 10, 13–15, 19, 21, 22, 29, 32, 36, 46, 52, 57 and 62. Analysed as a mixture of **8**, with matrix (4-hydroxy-α-cyanocinnamic acid).

least 8–9 cycles of acidic deprotection in oligonucleotide synthesis using a two-fold diluted standard solution of trichloroacetic acid in dichloromethane (ABI/Cruachem) and a reduced deprotection time (see Experimental). For analysis, it was easily released as **8** using 1–3% TFA in the same solution (~1–3 min). Higher concentrations of TFA, while removing the tags, cause some decomposition of polyethyleneglycol (PEG) chains, which sometimes leads to complex signals in the area of 350–500 Da when the samples are run without matrix. These peaks showed mass-differences of 14–16 DA, suggesting them to be CH_2 and CH_2O fragments of the PEG linker (data not shown). The same overwhelming presence of PEG decomposition products was observed in LDI-TOF analysis when individual beads were glued directly to the gold sample well using a very thin film of rubber glue. It is possible that trityl



Scheme 2. Encoding and decoding in combinatorial synthesis.

cations interact with the oxygen bridges of PEG chains, thus being trapped in them and decreasing desorption. The presence of matrix (in case of detritylated tags) solves this problem. At the same time, when derivatised with different amines, MMTr(NHS) (**2**)-based tags produced the second highest intensity signals (after DMTr(NHS) (**1**)) and gave reasonably clean peaks, even when mixtures of compounds were analysed (Fig. 2).

To introduce a tagging moiety during oligonucleotide synthesis (Scheme 3), we synthesised the non-nucleoside phosphoramidite synthon **7** based on a propanediol structure,[54] which provides similar reactivity to the standard A, C, G and T phosphoramidites. The MMTr(NHS)Cl (**2**) has reduced reactivity compared to both DMTrCl and DMTr(NHS)Cl (**1**), and it is important, when synthesising **7**, to carry out the tritylation reaction at low temperatures to prevent the formation of ester bonds between the excess of propanediol and the activated carboxyl group (data not shown). The phosphoramidite **7** was stable in acetonitrile solution at rt for at least 2 days.

We mixed ca. 3–6 mol% of **7** with standard A, C, G and T phosphoramidites prior to oligonucleotide synthesis, in a way similar to that described by Cho et al.[24] Assuming the stepwise yield of oligonucleotide synthesis to be about 99%, for an 8-mer library synthesised using **7** as a 5% additive to all bases, we would have ca 60% of all sites of the beads occupied by full length oligonucleotides in the final product. The concentration of the first tag (5% of all initial sites) would be about two-fold greater than that of the last tag (5% of the remaining 60% of the sites), which still makes it possible to detect all of them in the same mixture. Oligonucleotide synthesis was carried out on a 4-column ABI machine. After each oxidation step, the columns were removed and treated with different amines as described in the experimental section. No particular rationale was used when choosing the masses of tags to be used for the encoding of a particular base/position. But in principle, certain mass-ranges could be used to code for either a base type (i.e. all A are coded by tags ranging from 400 to 500 Da, all C by tags in the interval of 500–600 Da etc.) or, alternatively, the position of the base might be a determining factor (i.e. position #1, or a 3′-end, (A, C, G or T) is coded by mass-tags ranging from 400–420 Da, position #2- by 420–440 Da etc.).

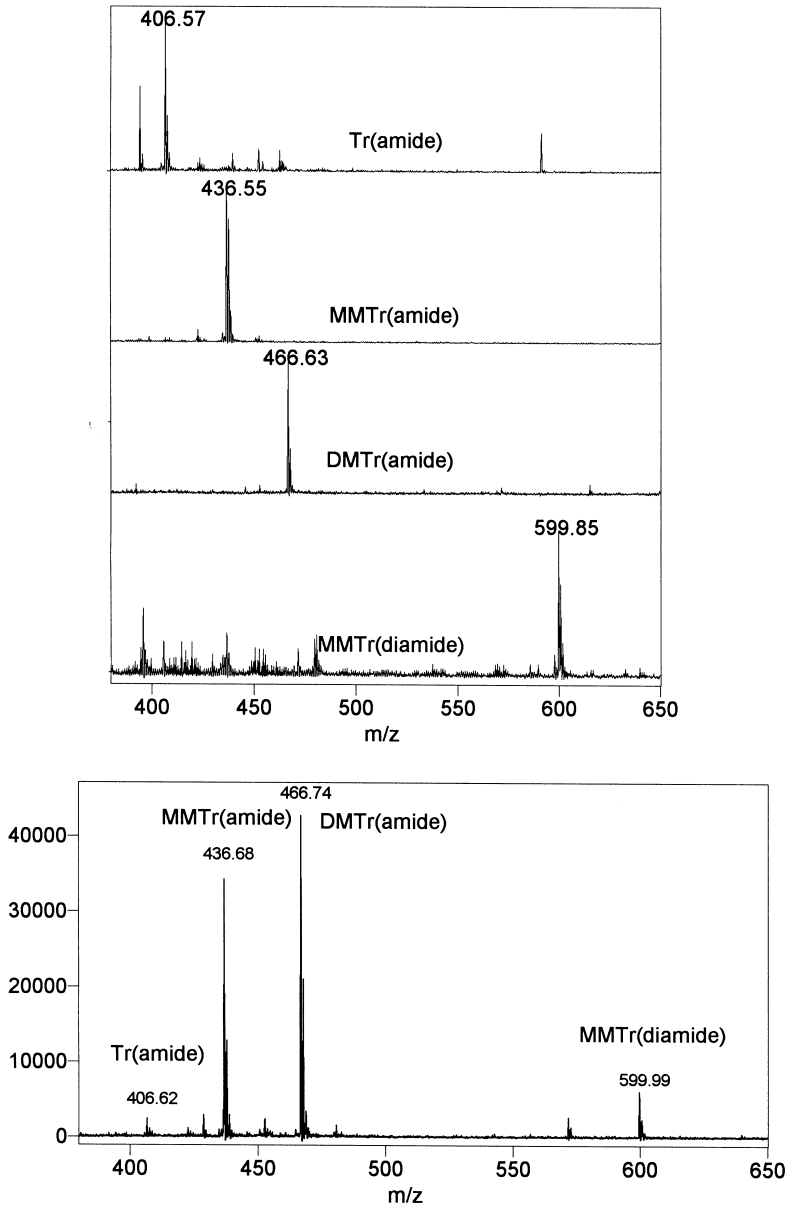
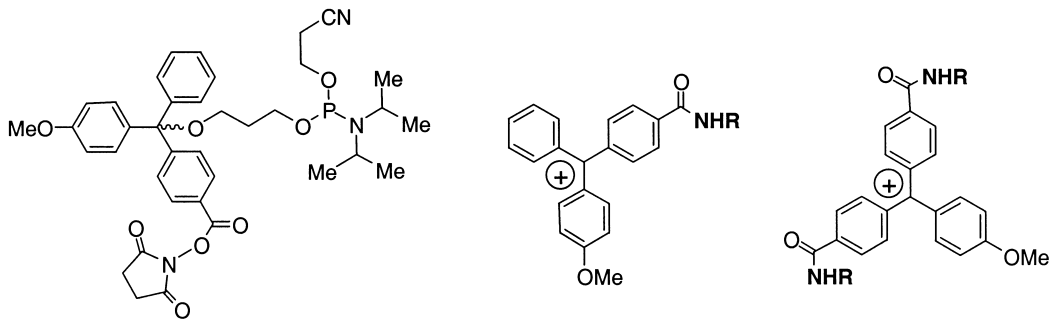


Figure 2. (Top) Individual mass-spectra of trityl-based non-purified pro-tags **1**, **2**, **3** and **6** (in the form of tritanols) treated with phenoxyethylamine (#35, Table 1); analysed without matrix (ionisation by laser irradiation). (Bottom) Mass-spectrum of a mixture of equimolar amounts of the same four mass-tags; analysed without matrix. (Expected masses of the tags: 406.6, 436.5, 466.5 and 599.9).



7 **8** **9**

Scheme 3.

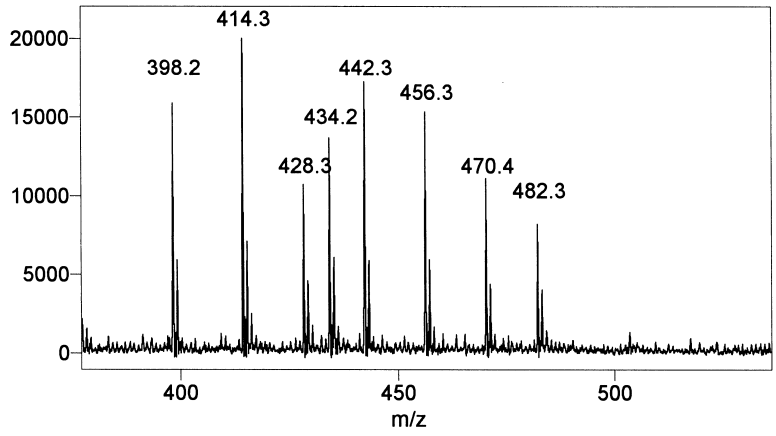


Figure 3. Decoding of the sequence of oligonucleotide attached to the beads selected from combinatorial library of 256 pyrine 8-mers by hybridisation to oligonucleotide 5′-Cy5-CTC.CTC.TC. The mass-tags shown encode for (from 3′-end): G (398), A (414), G (456), G (428), A (442), G (470), A (434) and G (482) (5′-GAG.AGG.AG).

Beads were selected by hybridisation with a Cy5-labelled oligonucleotide. The size of Rapp-beads (~0.3 mm) allows manual removal of positively identified beads, visible with the naked eye, from the pool. For smaller beads, automated methods such as flow-cytometry (FACS) might be used. Selected beads were detritylated and the mixtures of tags released analysed by mass-spectrometry (Fig. 3). The spectrum corresponded to the sequence of the oligonucleotide used for hybridisation.

To eliminate the problem of gradual loss of encoding MMTr-based tags **8** during the detritylation step in oligonucleotide synthesis, we have also used Fmoc as a 5′-protecting group,[57,58] thus omitting the use of acidic conditions altogether. After each oxidation step, the columns were removed from the synthesiser, and the beads were treated with the corresponding amines, washed with acetonitrile and then treated with 0.1 M DBU in acetonitrile[58] to remove Fmoc-protection. The tags encoding for 9-mer oligonucleotide synthesised using this strategy were detected using (MA)LDI-TOF analysis (data not shown). For longer sequences, the 3′-ethyl- or 3′-methylphosphoramidites of 5′-Fmoc-protected nucleosides could preferably be used instead of cyanoethylphosphoramidites, to prevent the loss of the cyanoethyl (Cnet) protecting group due to the treatment with amines and DBU.

Conclusions

We have shown that trityl-based mass-tags fly extremely well in the positive mode of an LDI process, presumably due to the formation of a highly stabilised trityl carbocation. By modifying the trityl moiety with a reactive group, the trityl-based pro-tag can be successfully used for encoding in combinatorial chemistry. Tags describing the sequence/composition of a biopolymer attached to an insoluble bead are introduced after each chemical transformation, by reacting the pro-tags with different primary amines. We have shown that the tags are easily detectable using (MA)LDI-TOF analysis, provided the mass-difference between them is at least 2–3 Da. In these conditions, the fragmentation of tritylamides is very low, which helps to identify the tags unambiguously. The scheme and reagents described here are very simple as compared to other methods of encoding. The tags are suitable for any combinatorial synthesis that does not involve strong acids, for example, peptide synthesis using Fmoc-protected amino-acids. An additional increase of the amount of reactive sites on the beads can be achieved by employing the 'trebling' phosphoramidite prior to combinatorial synthesis.[59,60] We were able to identify the oligonucleotide on a single bead selected from a hybridisation screen of a library of 256 sequences, thus demonstrating the efficiency and simplicity of our system, which is rapid, reliable and needs no special apparatus. The trityl tags could also be used to increase the desorbtion rate of compounds that are otherwise difficult to analyse in (MA)LDI-TOF.

Experimental

Split-and-mix synthesis of oligonucleotides was carried out in an Applied Biosystems 394 DNA/RNA four column synthesiser using standard phosphoramidite chemistry.[53] The solid support used was Tenta Gel Macrobeads OH, 280–320 μm, Rapp Polymer. Cy5 phosphoramidite was from Pharmacia/Biotech. UV-spectra were measured on a Spectronic 2000 spectrophotometer, Milton Roy Co., USA. MALDI- and LDI-TOF mass-spectra were recorded on a PE-ABI Voyager™ Elite Reflectron Delayed Extraction Instrument. Spectra were acquired with an accelerating voltage of 25 KV and 100 ms delay in the positive ion mode. Micrographs were taken on a Leica TCS NT confocal microscope equipped with an Ar/Kr multiline laser. ^{1}H NMR spectra were recorded on a Varian Gemini 200 200 MHz spectrometer. ^{31}P NMR spectra were recorded on a Brucker AC-500 spectrometer (internal standard: 80% phosphoric acid). HPLC was carried out on a Waters system (Milford, MA, USA). Chemicals were purchased from Aldrich Chemical Company (USA), Avocado Research Chemicals (UK), Lancaster Synthesis Ltd (UK), and Acros Organics (Fisher Scientific, UK). Phosphitylating reagent and oligonucleotide purification columns were from Sigma Chemical Company. Silica gel for column

chromatography, UV_{254} TLC plates and solvents were from BDH/Merck.

***N*-Succinimidyl-4-[bis-(4-methoxyphenyl)-chloromethyl]-benzoate (1).** It was synthesised according to the reported procedures.[40,41]

Racemic *N*-succinimidyl-4-[(4-methoxydiphenyl)-chloromethyl]-benzoate (2). This was synthesised according to reported procedures,[40,41] but 4-methoxybenzophenone was used in the Grignard synthesis instead of 4,4′-dimethoxybenzophenone. Total yield: 45%, yellow-white solid, mp 181–184°C. (Found: C, 66.58; H, 4.55; N, 3.20. $C_{25}H_{20}ClNO_5$ (M_W 449.88) requires C, 66.76; H, 4.48; N, 3.11%); ν_{max} (Nujol): 2919 (br), 2852, 1768, 1735, 1605, 1508, 1459, 1376, 1304, 1254, 1202, 1068 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 8.11 (d, 2H, J=8.4 Hz, OOC-aryl-H, *ortho*), 7.53 (d, 2H, J=8.4 Hz, OOC-aryl-H, *meta*), 7.31 (m, 5H, arom.), 7.15 (d, 2H, J=8 Hz, CH_3O-aryl-H, *meta*), 6.85 (d, 2H, J=8 Hz, CH_3O-aryl-H, *ortho*), 3.8 (s, 3H, OCH_3), 2.9 (s, 4H, NHS). ^{13}C NMR ($CDCl_3$, internal standard $CDCl_3$ at 77.03 ppm, δ): 169.54, 153.2, 131.04, 130.29 (d), 129.53 (d), 128.24 (d), 127.96, 113.47 (d), 55.31, 25.56. HRMS (LDI-TOF) for corresponding tritanol, *m/z* (rel. intensity): 414.3 (100), 333.4 (6), 317.7 (27), 289.3 (4). [Calculated exact mass for $C_{25}H_{20}NO_5$ ($MMTr(NHS)^+$): 414.13415; found: 414.13621 (4.9 ppm error)].

***N*-Succinimidyl-4-[bis-(phenyl)-chloromethyl]-benzoate (3).** This was synthesised according to the reported procedures,[40,41] but benzophenone was used in the Grignard synthesis instead of 4,4′-dimethoxybenzophenone. Yellow-white solid, mp 170–173°C. (Found: C, 68.88; H, 4.14; N, 3.09. $C_{24}H_{18}ClNO_4$ (M_W 419.86) requires C, 68.66; H, 4.32; N, 3.34%); ν_{max} (Nujol): 2920–2800 (br), 1765, 1735, 1605, 1500, 1455, 1375, 1315, 1250, 1200 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 8.1 (d, 2H, J=8.5 Hz, OOC-aryl-H, *ortho*), 7.51 (d, 2H, J=8.5 Hz, OOC-aryl-H, *meta*), 7.28 (m, 10H, arom.), 2.86 (s, 4H, NHS). ^{13}C NMR ($CDCl_3$, δ): 169.57, 146.12, 130.37, 128.42 (d), 127.95 (d), 123.85, 81.86, 33.77, 25.52. HRMS (LDI-TOF) for corresponding tritanol, *m/z* (rel. intensity): 401.1 (75), 384.1 (100), 286.2 (8), 259.6 (5). [Calculated exact mass for $C_{24}H_{18}NO_4$ ($Tr(NHS)^+$): 384.12358; found: 384.12375 (0.4 ppm error)]. The same product was obtained using commercially available phenylmagnesium bromide and 2-(4-benzophenyl)-4,4-dimethyl-1,3-oxazoline (**4**) in the Grignard reaction.

2-(4-Benzophenyl)-4,4-dimethyl-1,3-oxazoline (4). 4-Benzoylbenzoic acid (50 g, 0.22 mol) was refluxed in thionyl chloride (300 ml) for 3 h, evaporated (the product crystallises from the oil) and then evaporated with toluene (2×30 ml). The residue was dissolved in dry methylene chloride (250 ml). To this ice-cooled solution, 2-amino-2-methylpropan-1-ol (46 g, 0.51 mol) in dry methylene chloride (150 ml) was added dropwise for 2 h. The solution was stirred overnight at rt, and the precipitate was washed several times with methylene chloride. Combined fractions were evaporated, slowly dissolved in thionyl chloride (350 ml) and refluxed for 4 h. The reaction mixture was evaporated to one-third volume, poured into dry ether (2 l) and kept overnight at 4°C. The hydrochloride precipitate was dissolved in water (1 l) at 10°C, and 5 M KOH (300 ml) was added with stirring. The mixture was extracted with chloroform (3×350 ml), the organic phase dried over $CaCl_2$ and evaporated. The product was crystallised from toluene to give 42 g (75%) of white crystalline solid, mp 86–88°C. (Found: C, 77.68; H, 5.95; N, 4.82. $C_{18}H_{17}NO_2$ (M_W 279.33) requires C, 77.40; H, 6.13; N, 5.01%); ν_{max} (Nujol): 2923 (br), 2854, 1654, 1637, 1463, 1376, 1311, 1280, 1069 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 8.1 (d, 2H, J=8.5 Hz, O(N)C-aryl-H, *ortho*), 7.8 (m, 4H, arom.), 7.6 (m, 1H, arom.), 7.51 (m, 2H, arom.), 4.18 (s, 2H, CH_2), 1.45 (s, 6H, CH_3). ^{13}C NMR ($CDCl_3$, δ): 132.89, 130.1 (d), 128.53 (d), 79.34, 67.88, 28.25. HRMS: MALDI-TOF (α-cyano-4-hydroxycinnamic acid): 279.1 (MI), 302.1 ($MI+Na^+$), 319.7 ($MI+K^+$). LDI-TOF, *m/z* (rel. intensity): 279.1 (100), 202.1 (12), 181.1 (25). [Calculated exact mass for $C_{18}H_{17}NO_2$: 279.12593; found: 279.12614 (0.8 ppm error)].

4,4′-[bis-(2-(4,4-Dimethyl-1,3-oxazolyl))]-4″-methoxytritanol (5). To magnesium turnings (1.5 g) activated with iodine bromophenyl oxazoline (15.34 g, 0.06 mol) in dry THF (150 ml) and a catalytic amount of RED-AI® and MeI were added with stirring and the mixture was refluxed for 3 h, cooled to rt and methyl 4-methoxybenzoate (4.64 g, 28 mmol) in dry THF (40 ml) was added dropwise. The mixture was gently refluxed for 6 h, cooled to rt and water (10 ml) was added with stirring. The organic phase was carefully decanted and the residue washed several times with small portions of THF. Combined organic fractions were evaporated and purified (flash-chromatography) to give 11.4 g (84%) of light yellow solid. (Found: C, 74.57; H, 6.57; N, 5.59. $C_{30}H_{32}N_2O_4$ (M_W 484.59) requires C, 74.36; H, 6.66; N, 5.78%); ν_{max} (Nujol): 2950–2800 (br), 1714, 1648, 1607, 1509, 1462, 1408, 1354, 1316 (br), 1253 7.85 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 8.11 (d, 4H, J=8.4 Hz, O(N)C-aryl-H, *ortho*), 7.32 (d, 4H, J=8.4 Hz, O(N)C-aryl-H, *meta*), 7.12 (d, 2H, J=8 Hz, CH_3O-aryl-H, *meta*), 6.81 (d, 2H, J=8 Hz, CH_3O-aryl-H, ortho), 4.12 (s, 4H, CH_2), 3.78 (s, 3H, OCH_3), 1.37 (s, 12H, CH_3). ^{13}C NMR ($CDCl_3$, δ): 162.15, 159.1, 150.1, 138.64, 131.5 (d), 129.15 (d), 128.01 (d), 126.95, 113.51 (d), 81.38, 79.13, 67.52, 55.18, 28.21. HRMS: MALDI-TOF (α-cyano-4-hydroxycinnamic acid): 484.9 (MI), 467.6 ($MI-OH$). LDI-TOF, *m/z* (rel. intensity): 484.2 (100), 467.2 (43), 453.2 (7), 369.2 (7.5), 310.1 (36), 294.1 (20). [Calculated exact mass for $C_{30}H_{32}N_2O_4$: 484.23621; found: 484.23835 (4.4 ppm error)].

4,4′-[bis-(2-(Succinimidylcarboxy)]-4″-methoxytrityl chloride (6). The solution of **5** (10 g, 21 mmol) in 80% acetic acid (250 ml) was kept at 72°C for 48 h, evaporated and then re-evaporated from water (2×50 ml). The product was dissolved in 50% EtOH/water (75 ml), refluxed for 3 h and evaporated to one-third volume. The mixture was then dissolved in water (100 ml) and acidified with 3 M HCl to pH 1–2. The precipitate was dissolved in chloroform, dried (Na_2SO_4) and evaporated to dryness and additionally dried in vacuo overnight. The resulting dicarboxylic acid was dissolved in dry THF (100 ml). *N*-hydroxysuccinimide (8.5 g, 74 mmol) was added and the mixture was cooled to 0°C. Dicyclohexylcarbodiimide (8.5 g, 41 mmol) in dry THF (20 ml) was added dropwise with stirring. The reaction

mixture was stirred for 1 h at 0°C and then overnight at rt. Dicyclohexylurea was filtered off and organic phase was evaporated to dryness and purified (flash-chromatography) to give 8.5 g (72%) of yellow-white solid, mp 212–215°C (decomp.). (Found: C, 63.13; H, 4.04; N, 4.88. $C_{30}H_{24}N_2O_{10}$ (M_W 572.52) requires C, 62.94; H, 4.23; N, 4.89%); ν_{max} (Nujol): 2950–2800 (br), 1765, 1736, 1606, 1510, 1460, 1406, 1375, 1255, 1200 (br), 1070 cm^{-1}. ^1H NMR ($CDCl_3$, δ): 8.11 (d, 4H, J=8.5 Hz, OOC-aryl-H, *ortho*), 7.5 (d, 4H, J=8.5 Hz, OOC-aryl-H, *meta*), 7.11 (d, 2H, J=8.1 Hz, CH_3O-aryl-H, *meta*), 6.88 (d, 2H, J=8.1 Hz, CH_3O-aryl-H, *ortho*), 3.81 (s, 3H, OCH_3), 2.9 (s, 8H, CH_2). ^{13}C NMR ($CDCl_3$, δ): 169.69, 161.86, 159.5, 153.6, 137.64, 130.56, 129.4, 128.47, 124.21, 113.85, 81.34, 55.32, 25.55. HRMS: MALDI-TOF (α-cyano-4-hydroxycinnamic acid): 554.7 ($MMTr(2NHS)^+$), 570.8 (MI). LDI-TOF, *m/z* (rel. intensity): 572.1 (100), 554.4 (45), 474.1 (10), 397.8 (5). [Calculated exact mass for $C_{30}H_{24}N_2O_{10}$: 572.14310; found: 572.14498 (3.3 ppm error)]. This compound was converted into the corresponding trityl chloride by refluxing in AcCl/toluene (100 ml) for 3 h. The reaction mixture was then evaporated to one-third volume. Toluene (60 ml) was added, the mixture was again evaporated to one-third volume and used without further purification.

***O*^{1}-{[4-(Succinimidylcarboxy)]-4′-methoxytrityl}-1,3-propanediol.** The title compound was synthesised from **2** according to a published procedure,[54] but the tritylation step was carried out in pyridine at 0°C overnight without a catalyst. Monoprotected propanediol was obtained in 55% yield after purification by flash-chromatography as a white foam. (Found: C, 68.45; H, 5.81; N, 3.07. $C_{28}H_{27}NO_7$ (M_W 489.52) requires C, 68.70; H, 5.56; N, 2.86%); ^1H NMR ($CDCl_3$, δ): 8.10 (d, 2H, J=8.3 Hz, arom.), 7.6 (d, 2H, J=8.3 Hz, arom.), 7.29 (m, 5H, arom.), 7.14 (d, 2H, J=8 Hz, arom.), 6.89 (d, 2H, J=8 Hz, arom.), 3.8 (s, 3H, OCH_3), 3.79 (t, 2H, J=6 Hz, CH_2OH), 3.28 (t, 2H, J=6 Hz, $MMTr(NHS)OCH_2$), 2.9 (s, 4H, succinimide), 1.89 (quin, 2H, J=6 Hz, $CH_2CH_2CH_2$), 1.65 (br.s., 1H, O*H*). ^{13}C NMR ($CDCl_3$, δ): 62.5 (*C*1), 61.02 (*C*3), 55.02 (OCH_3), 32.67 (*C*2), 25.12 ($C(O)CH_2CH_2C(O)$). HRMS: MALDI-TOF (α-cyano-4-hydroxycinnamic acid): 489.1 (MI), 527.9 ($MI+K^+$); LDI-TOF, *m/z* (rel. intensity): 489.3 (25), 414.4 (100). [Calculated exact mass for $C_{28}H_{27}NO_7$: 489.17875; found: 489.17906 (0.6 ppm error)].

***O*^{1}-{[4-(Succinimidylcarboxy)]-4′-methoxytrityl}-*O*^{3}-(*N*,*N*-diisopropylamino-2-cyanoethoxyphosphinyl)-1,3-propanediol (7).** Phosphitylation of monoprotected propanediol was carried out as described[54] to give the title phosphoramidite as a white foam in 70% yield. (Found: C, 64.75; H, 6.22; N, 5.86. $C_{37}H_{44}N_3O_8P$ (M_W 689.73) requires C, 64.43; H, 6.43; N, 6.09%); ^1H NMR ($CDCl_3$, δ): 8.11 (d, 2H, J=8.5 Hz, arom.), 7.59 (d, 2H, J=8.5 Hz, arom.), 7.31 (m, 5H, arom.), 7.15 (d, 2H, J=8.4 Hz, arom.), 6.88 (d, 2H, J=8.4 Hz, arom.), 3.81 (s, 3H, OCH_3), 3.7–3.3 (m, 6H, $NCCH_2CH_2OP$, CH_2OP and $CH(CH_3)_2$), 3.2 (t, 2H, J=6 Hz, $MMTr(NHS)OCH_2$), 2.9 (s, 4H, succinimide), 2.65 (m, 2H, CH_2CN), 1.93 (quin, 2H, J=6 Hz, $CH_2CH_2CH_2$), 1.16 (d, 12H, J=6 Hz, $CH(CH_3)_2$). ^{31}P NMR ($CDCl_3$, δ, md): 144.512. HRMS (MALDI-TOF): calculated exact mass for $C_{37}H_{44}N_3O_8P$: 689.28660; found: 689.28799 (2 ppm error).

Analysis of trityl-based tags

0.1 M solutions of compounds **1**, **2**, **3** and **6** were prepared in a mixture of THF/dioxane (1:1). 110 μl of these solutions were mixed with 40 μl (80 μl in case of **6**) of 0.5–1 M solutions of different amines (Table 1) as described by Gildea[40] and Coull.[41] The mixtures were then analysed with and without matrix either directly or when mixed in different combinations.

Oligonucleotide synthesis

Oligonucleotide synthesis was carried out using commercially available standard A, C, G and T, PAC, fluorescein and Cy5 phosphoramidites according to manufacturer's protocols.

Synthesis of the combinatorial library

Phosphoramidite **7** was added to standard A, C, G and T phosphoramidites or to 5′-Fmoc-phosphoramidites[57] up to 3–6 mol.% of total amount of phosphoramidite. 35–40 mg (ca. 3500 beads) of Rapp TentaGel Macrobeads were placed in each of four polypropylene DNA synthesis columns (1 μmol scale, Glen Research, USA). The oligonucleotide synthesis was carried out on 1 μmol scale using phosphoramidite mixtures: A+**7**, C+**7**, G+**7** and T+**7**, according to manufacturer's protocol, but the supply of deblocking reagent (diluted to 50% of its original concentration with methylene chloride) to the columns was reduced to 10–15 s, with a subsequent waiting step (10 s) followed by another portion of acid (10 s). Subsequent thorough acetonitrile washing of the columns ensured that all $DMTr^+$ is desorbed. Before each detritylation step, the columns were washed with acetonitrile using Manual Control mode, and then treated with corresponding amines (0.3–0.5 ml of 0.5–1 M solutions in dioxan/THF, depending on the solubility of an amine) for 1 min using 1 ml syringes. The columns were then washed exhaustively with acetonitrile and dried in vacuo for 15 min. The beads from all columns were then combined together in a 0.3 ml reaction vial with conical chamber (Pierce), mixed, and split again in four portions by pipetting the suspension of beads in acetonitrile (about 1 volume of solvent per 1 volume of beads) using a 1 ml Eppendorf micropipette tip. The procedure was repeated until the end of the synthesis. The beads were then washed, dried, deprotected for 14 h in concentrated ammonia (1.5 ml) at 55°C, then washed several times with distilled water, dried and stored at 4°C.

Hybridisation of the combinatorial library and detritylation

The beads were hybridised to 5′-Cy5-labelled 8-mer oligonucleotide 5′-CTC.CTC.TC in 1.5 ml of 3.5 M TMA buffer[55] in a 4 ml vial which was rotated on a Spiramix 10 machine overnight at 8°C. The beads were then washed five times with TMA buffer at the same temperature, transferred on to the surface of a 7.5×5 cm microscope slide and the excess of the buffer removed by blotting with tissue paper. Coloured or otherwise identified beads were then removed using forceps (usually about 10–15 beads), washed with water, acetone and dried. The trityl tags were cleaved by

treating the beads with 10–50 μl of 1–3% (v/v) solution of trifluoroacetic acid in standard Deblok Solution (Cruachem; trichloroacetic acid in dichloromethane) for 3–4 min. The supernatant was evaporated several times with acetone or methanol to remove the acids and the residue was analysed by (MA)LDI-TOF HRMS.

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