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The Preparation And Synthetic Application Of Heterobifunctional Biocompatible Spacer Arms[1,2]

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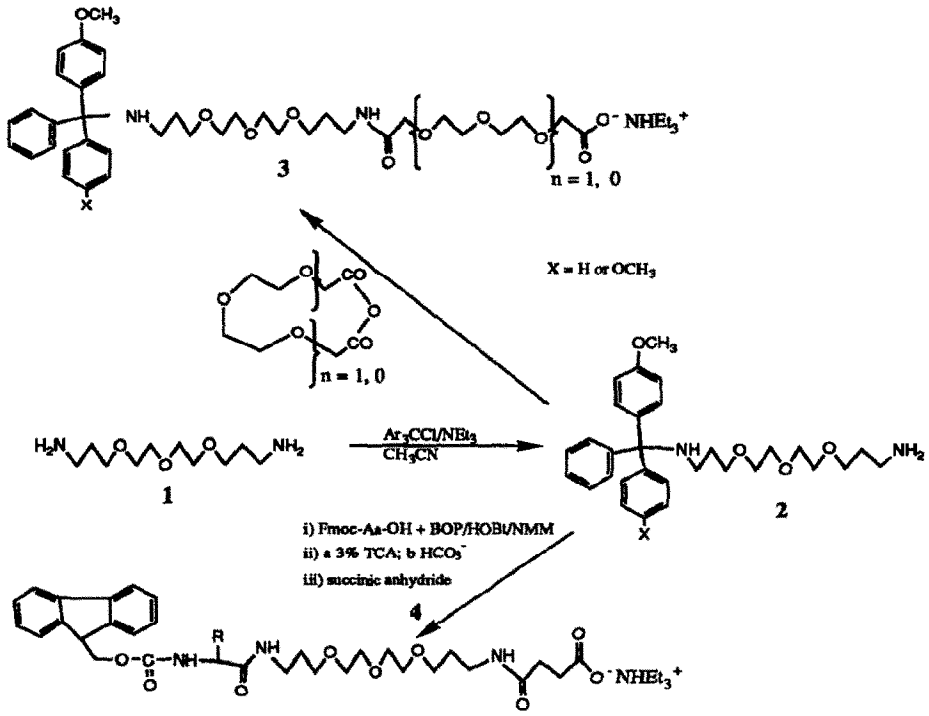
Abstract: Simple spacer arm molecules which provide improved biomolecule synthesis and display are described. Functionalization with non-polar triaryl groups confers organic solvent solubility and facilitates isolation.

It is well established that long (greater than 20 Å) spacer or linker arms are required for efficient display of haptens attached to carriers or to solid support surfaces. These minimize steric hindrance and environment effects imposed by the surface or carrier. Ligands may be either assembled on the support, in pure form, or as deliberate mixtures, or may be preassembled and subsequently attached. Polyoxyethylene provides a flexible, chemically inert, biocompatible basis for linker arms. The preparation of such long-chain heterobifunctional derivatives is complicated both by difficulty in separation of products from homobifunctional impurities and starting materials; and by the polydisperse nature of the polyethylene glycol product derivatives. Ion exchange chromatography provides one route.[3]

This report describes the modification of 4,7,10-trioxa-1,13-undecanediamine (**1**, a readily available intermediate used in azacrown ether synthesis[4]) to give intermediate length defined spacer arms. Use of non-polar triaryl protecting groups (Scheme 1) ensures that the desired intermediates and products have adequate solubility in water immiscible organic solvents, from which starting materials and byproducts may simply be removed by aqueous extraction or short column chromatography.[5] After reaction of p-anisoyl-diphenylmethane chloride (MMT-Cl), or 4,4'-dimethoxytrityl chloride (DMT-Cl), with excess **1**, intermediates **2** were succinylated, either directly, or after work-up, to give the desired linkers **3** (n = 0).[6,7] Alternatively, intermediates **2** may be reacted with any suitably protected amino acid derivative (Fmoc is preferred, but Boc and other non-polar groups stable to mild acid may be useful). Removal of the MMT or DMT groups from the intermediate with 3% trichloroacetic acid in dichloromethane, neutralization by bicarbonate washing and succinylation in acetonitrile gave **4** in good yield after chromatographic purification. The method has been applied with a variety of amino acids; L-*nor*leucine provides an internal reference amino acid (4Nle), whereas Lys(Fmoc), Lys(Boc), and Lys(Aloc) give linkers (4KF, 4KB & 4KA, resp.) which may be coupled in repeated addition cycles to achieve linear or branched dendritic display of identical or different ligands. Preparation of linkers **4** by direct coupling of Fmoc-amino acids to **1** could be achieved, but was less satisfactory as excess diamine lead to unexpectedly rapid Fmoc removal.[8]



Scheme 1. *Synthesis of alkyloxytrityl and Fmoc protected spacer arm derivatives*

Derivatives 3 and 4 have been coupled in excellent yield to a variety of amino-functionalized substrates using the efficient BOP+ HOBt method.[9] Coupling of 3 (n = 0, 2.5 equiv.) to 1000Å aminopropylCPG[10] occurred in ca. 99% yield giving CPG I with a 30 Å spacer arm.[11] After MMT removal, and base wash, a further high efficiency coupling gave CPG II with a doubled 60Å spacer. These supports have been used in the routine synthesis of a variety of medium length oligonucleotides (up to 70 residues) and small peptides (<10 residues). Data regarding product purity and initial coupling efficiencies in DNA synthesis (Table 1) showed that significantly improved coupling can be obtained with the single and doubly linker modified supports.

Table 1. *Comparison of T-Amidite Coupling Efficiency to T-Derivatized CPG Supports*

CPG Type	Spacer Added (Å)	Loading (μmol/g) DMT-T-succinate	Coupling Efficiency (%)*
Amino-Propyl	None	36	80±3
CPG I	30	34	90±2
CPG II	60	33	97±2
LCAA-CPG**	?	31	85±3

* Coupling efficiency average of first 2 addition cycles, 3 determinations each; performed on Biosearch Model 8700 synthesizer with 0.25 μmol columns, amidite concentration at 20 mg/mL. ** spacer undefined, supplied by Glen Research, Sterling VA.

Regarding peptide synthesis, a side-by-side comparison was made of the yields and product purities obtained in the preparation of Leu^5 enkephalin on hydroxymethylbenzoyl^{12} functionalized CPG's (reverse-phase HPLC profiles are depicted in Figure 1).[13] Use of the doubly linked 60Å spacer CPG II support raised the product yield to 70% from the 35% obtained with aminopropyl CPG. The major impurities in syntheses on aminopropyl CPG (C, D, were almost eliminated with the spacer arm support (A, B). Additionally, coupling in acetonitrile with the improved support gave at least as good results as with DMF as solvent, whereas, with the parent aminopropyl functionalized CPG, acetonitrile provided a less effective medium. Of further interest is, that with this combination of support and handle, TFA mediated side-chain deprotection could be performed (without significant loss) prior to mild base cleavage, a procedure which greatly simplifies product isolation and purification.

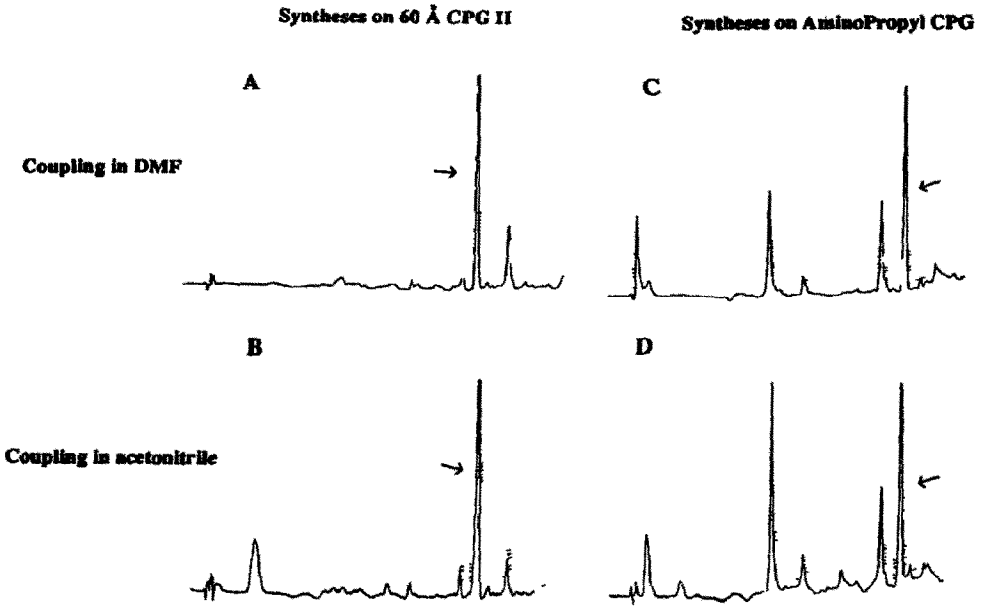


Figure 1. *Simultaneous syntheses of YGGFL on hydroxymethybenzoyl linked Control Pore Glass supports, 30 minute couplings perfomed with the BOP/HOBt/NMM method, deprotection with 95% TFA/5% H_2O for 2 hours, cleavage with 0.1M KOH in 68% dioxan/water for 30 minutes.*

An alternative and preferred route to longer spacer arm functionalized materials is provided by reaction of 3,6,9-trioxaundecanedioic acid anhydride with 2 (X = H) to give 3 (n = 1).[14] This extended version couples as efficiently as its shorter variation, and obviates the tedious MMT removal, base wash and recoupling procedures which become unwieldy when performed on large scales.

In conclusion, a variety of spacer arm molecules and useful derivatized support materials have been prepared which allow efficient biomolecule assembly, and which will facilitate accessible display of ligands for diagnostic, affinity chromatographic, library and analytical applications.[15]

References and Notes

1. Application of analogous spacer arms in the preparation of branched peptide/DNA libraries has recently been reported; Nielsen, J.; Brenner, S.; Janda, K.D. *J.Amer.Chem.Soc.* **1993**, *115*, 9812-9813.
2. Abbreviations follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J.Biol.Chem.* **1972**, *247*, 977-983. Additionally, the following abbreviations are used: Aloc, allyloxycarbonyl; Boc, *t*-butyloxycarbonyl; BOP, benzotriazolyloxytris(dimethylamino)-phosphonium hexafluorophosphate; CPG, controlled pore glass; DMF, dimethylformamide; DMT, 4,4'-dimethoxytrityl; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; LCAA, Long Chain Alkyl Amine; MMT, 4'-monomethoxytrityl; TCA, trichloroacetic acid; TLC, thin layer chromatography.
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4. Krakowiak, K.E.; Bradshaw, J.S. *J. Org. Chem.* **1991**, *56*, 3723-3725.
5. Jaschke, A.; Furste, J.E.; Cech, D.; and Erdmann, V.A. *Tetrahedron. Lett.* **1993**, *34*, 301-304 describe a related method for the production of DMT-polyethylene glycol-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidites, and related derivatives, used in the preparation of 3' and 5' polyethylene glycol modified synthetic nucleotides.
6. Monomethoxytrityl chloride (Aldrich, 15.5 g, 50 mmol) dissolved in methylene chloride (100 mL) was added dropwise to a rapidly stirred solution of 4,7,10-trioxa-1,13-tridecanediamine (Fluka, 22.6 g, 100 mmol) in 1:1 acetonitrile/pyridine (200 mL). After the addition was complete, succinic anhydride (30 g, 0.3 mole) was added and the succinylation stirred for 3 hours at room temperature. The solvents were removed by rotary evaporation *in vacuo*, and the resulting gum dissolved in methylene chloride (500 mL). The solution was washed twice with water, once with brine, then concentrated *in vacuo* to ca. 50 mL. This was applied to a column of silica gel (250 g) equilibrated in methylene chloride containing 0.5% triethylamine. The column was eluted with a step-wise gradient from 0 to 15% methanol, the product 3 (n = 0), a clear gum (19.6 g, 56%) being isolated from fractions eluting with 10% methanol. The overall yield may be improved by isolating the side-product bis-MMT diamine (inevitably formed, no matter what stoichiometry or mixing employed, because of the high reactivity of the MMT-Cl), and treating it carefully with trichloroacetic acid to generate more 2 (X = H).
7. DMT variants are preferable in sensitive or large scale applications allowing more rapid deprotection than with MMT; acidic conditions must be avoided through out work up or subsequent coupling.
8. Fmoc removal rate comparable to that provided by piperidine.
9. Hudson, D. *J. Org. Chem.* **1988**, *53*, 617-624.
10. Dry CPG (423 g; Bioran from Schott-Gerate Gmbh, D-65719 Hofheim, Germany; pore size 99.6 nm, grain size 130-250 μm, surface area 31 m^2/g) in acetonitrile (1.2 L) was reacted with gentle agitation with aminopropyltriethoxysilane (5.2 mL) at 50° C for 2 hours. Bis(trimethylsilyl)-acetamide (40 mL) and pyridine (4 mL) were added and gentle agitation continued at room temperature overnight. After extensive washing 430 g of aminopropyl CPG loaded at 42 μmol/g were obtained.
11. 3 (n = 0) (30 g, 45 mmol) in 0.3M N-methylmorpholine in acetonitrile (250 mL) was treated with BOP (23 g, 50 mmol) and HOBt (6.8 g, 50 mmol). After 5 minutes acetonitrile (1 L) and aminopropyl-CPG (440 g, 55 μmol/g) were added and the slurry swirled gently overnight. The material was filtered, washed with acetonitrile (1 time), then treated with acetic anhydride/lutidine/N-methyl imidazole/THF (10:10:17:163; 1 L) for 10 minutes. Extensive washing and drying gave CPG I (445 g). CPG II was prepared identically by recoupling of 3 (n = 0) after MMT removal, and base wash (both performed by standard procedures).
12. Atherton, E.; Logan, C.J.; Sheppard, R.C. *J. Chem. Soc. Perkin I* **1981**, 538-546.
13. Waters Delta Pak C-18 column eluted over 35 minutes at 1.5 mL/min with a linear gradient from 0.1M ammonium acetate pH 6.5 to 1:1 this buffer in acetonitrile. Product (arrowed) eluting at ca. 18 minutes confirmed by HPLC/electrospray M/S (m+H = 556.6, theoretical = 556.62); impurities (especially obvious in C, D) have not been identified but are not simple failure sequences.
14. Dicyclohexylcarbodi-imide (1.05 g, 5 mmol) was added to a stirred solution of 3,6,9-trioxaundecanedioic acid (Fluka, 1.2 g, 5 mmol) in dichloromethane (20 mL). The reaction was left overnight and the precipitated urea removed by filtration. The filtrate was added to 2 (X = H, 1.7 g, 3.4 mmol) and triethylamine (0.7 mL, 5 mmol) and stirred overnight. TLC (methanol /dichloromethane, 1:9 + 0.5% acetic acid) showed complete reaction. The solution was evaporated and the residue purified on silica gel (60 g) as in ref. 6 to yield 3 (n = 1, X = H), 2.15 g (gum, 79%).

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