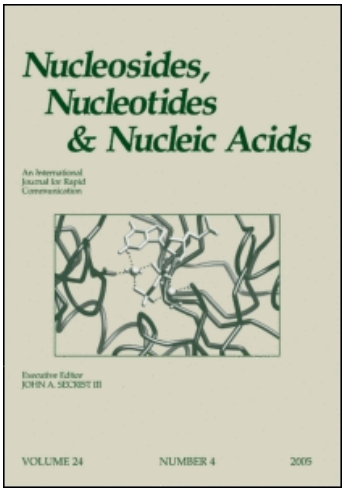

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Silica Gel Functionalised with Different Spacers as Solid Support for Oligonucleotide Synthesis

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SILICA GEL FUNCTIONALISED WITH DIFFERENT SPACERS AS SOLID SUPPORT FOR OLIGONUCLEOTIDE SYNTHESIS

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Abstract. The phosphotriester method for synthesis of oligonucleotides has been used for evaluating silica with spacer groups. Three different spacers were synthesized of which a long chain alkylamine, a double glycine and a double γ-aminobutyric acid unit. Only the double glycine spacer improved the yields of the first condensations and is now routinely used in our laboratory.

INTRODUCTION

Phosphotriester synthesis of oligodeoxynucleotides on a silica support is by now a well established method[1-3] and can be performed on either an automated system or a bench top synthesizer with a continuous column procedure. This is possible because of the rigidity of the structure, the low resistance and low swelling capacities. Cycling times and wash procedures are shortened and the support is easier to handle.

Looking at the yield per cycle - determined by spectrophotometric analysis - we noticed a well diminished yield for the first cycle and a slightly diminished one for the second as compared to further cycles. This can also be seen in published results of triester synthesis on silica[1-3].

It seemed interesting to try to improve these yields by putting in different spacers to prevent possible steric constraints or repulsion effects. Three different spacers were synthesized and their influence on the yields of the first cycles were compared to those on a conventional aminopropyl silica 1 : a silica with a long aliphatic chain 2 and aminopropyl silica with either two supplementary glycine 3 or γ-aminobutyric acid unities 4.

1	$-Si-(CH_2)_3NH_2$	8
2	$-Si-(CH_2)_{11}NH_2$	16
3	$-Si-(CH_2)_3NH(COCH_2NH)_2H$	14
4	$-Si-(CH_2)_3NH(COCH_2CH_2CH_2NH)_2H$	18

The spacer between the silica surface and the first nucleoside contains respectively 8, 16, 14 and 18 atoms.

RESULTS AND DISCUSSION

Synthesis of the long chain alkylamine. In analogy with the preparation of aminopropyl silica[4] (see also experimental part), 11-aminoundecyl-triethoxysilane had to be synthesized. Undecylenic acid was converted to the amide via the acid chloride. Reduction of the amide function gave ω-undecylenylamine 5a. Tritylation of 5a with chlorotriphenylmethane in pyridine at 100°C gave 6a. In the same way allylamine was derivatised to 6b. The addition reaction of triethoxysilane (1,8 eq.) in the presence of hexachloroplatinate as the catalyst (10^{-3} to 10^{-4} eq.) was carried out at room temperature during 15 h (yield 72,5 %). A side reaction is the reduction of the double bond to 7. This reduction is enhanced by heating the mixture, by higher concentrations of H_2PtCl_6 and by the presence of triphenylphosphine. In the absence of the chloroplatinate neither addition nor reduction took place. In contrast this reduction was not observed when 6b was reacted with triethoxysilane/-H_2PtCl_6, and therefore this reaction was carried out at elevated temperature (85°C) and for a shorter time (2 h - 90 % yield).

The trityl group was left on the silylating agent 8a,b as a marker for measurement of the amount of bound spacer groups (hydrolysis with $HClO_4$ 70 % - EtOH 3:2 and UV-spectrophotometric measurement at 430 nm, = 40,000). Apparently the trityl group prevents the reaction with silica gel to a great extent : all our trials were either negative or resulted in a very low functionality (max. 15 µmol NH_2/g silica). This was the case with the reaction performed as well in boiling toluene[5] as in $EtOH/H_2O$[4].

Therefore functionalisation of the silica was repeated with 11-aminoundecyl-triethoxysilane 9a. The product can be synthesized in two dif-

ferent ways. Hydrogenolysis (H_2/Pd or better H_2/Pd(OH)$_2$ in EtOH) of the trityl derivative 8a and purification on XAD yielded 82 % 9a. Alternatively one can hydrosilylate directly 5a. Hydrosilation of allyl amine with triethoxysilane in the presence of H_2PtCl_6 is a well known reaction in the industry. The resulting 3-aminopropyltriethoxysilane 9b is used to modify silica's for HPLC and TLC. Allylamine reacts slower than the corresponding olefines without an amine function[6]. Under the same conditions undecylenylamine 5a reacts poorly[7]. Prolonged heating is necessary and one gets a complex mixture resulting from silylation of the amine, hydrosilylation of the double bond, a combination of both and polymerisation (telomerisation). Therefore a catalytic amount of triphenylphosphine (2.10^{-4} eq.) was added. The reaction in this case was fast and selective (84 % yield after 4 h at 130°C). The favourable influence of triphenylphosphine on the reaction was already mentioned by Vybiral and all[8].

Untreated silica was reacted with 9a by shaking during 2 h in 95 % EtOH, filtration and heating at 110°C during 1 h. The functionality was determined by reaction with p-nitrobenzoylchloride, hydrolysis of the amide with 2 N NaOH and measurement of the absorption of the p-nitrobenzoate at 275 nm (see Table 1). The more laborious determination of the functionality through succinylation of the support and subsequent esterification with p-nitrophenol[9] gives the same results.

Incorporation of amino acids as spacer group. The introduction of amino acids on the solid support can be done by condensing the acid function of the amino acid with aminopropyl silica 1. The amine was protected with a 9-fluorenylmethyloxycarbonyl (FMOC)-group[10], while the carboxyl function was activated by reaction with DCC in CH_2Cl_2 to form the symmetric anhydride. The reaction with 1 was performed in DMF[11]. Alternatively, the N-FMOC-amino acid 10a,b (8 eq.) can be condensed with 1 by in situ activation with DCC in pyridine. Both methods gave similar results. After blocking the remaining aminopropyl functions with acetic anhydride in pyridine (1 h), the N-protecting group was hydrolyzed with piperidine 25 % in DMF in 10'[11]. Spectrophotometric measurement at 300 nm of the formed N-(9-fluorenylmethyl)piperidine 11 gives us the amount

TABLE 1. Functionality of alkylamine silica

Silica (pore size)	aminoundecyl silica	aminopropyl silica
Fractosil 1000 (1400 A)	-	65
Fractosil 500 (420 A)	70	250
Fractosil 200 (219 A)	-	490
Silica G (60 A)	135	930

Aminoalkyl silica were prepared according to the experimental part. The figures give the incorporation of amine groups in μmol/g on silica with different pore size, determined by the p-nitobenzoylchloride method.

TABLE 2. Incorporation of amino acids

Silica	First unit*		Second unit*	
	A	B	A	B
Fractosil 1000 (65 μmol NH_2/g)	31	43	22	33
Fractosil 500 (250 μmol NH_2/g)	86	144	80	126

The amount of incorporation of amino acids in μmol/g support is shown. This was determined by hydrolysis of the FMOC-group and spectrophotometric measurement. A = Glycine; B = γ-aminobutyric acid; *reaction time for incorporation of the first unit was 15 h, for the second 2 h. Longer reaction didn't improve the amount of incorporation.

TABLE 3. Condensation cycle.

Nitromethane wash	3 min
$ZnBr_2$ in nitromethane-1 % water	15 min
Nitromethane wash	3 min
Pyridine wash	5 min
Coupling mixture	45 min
Pyridine wash	1 min
Capping mixture	10 min
Pyridine wash	3 min

The coupling mixture consists of 30 μmol monomethoxytritylnucleoside-phosphodiester, MSNT 200 μmol and 30 μl N-methylimidazole[14] in 300 μl pyridine. Capping is done with Ac_2O-DMAP-pyridine 1:0.05:9[14].

Long chain alkylamine synthesis

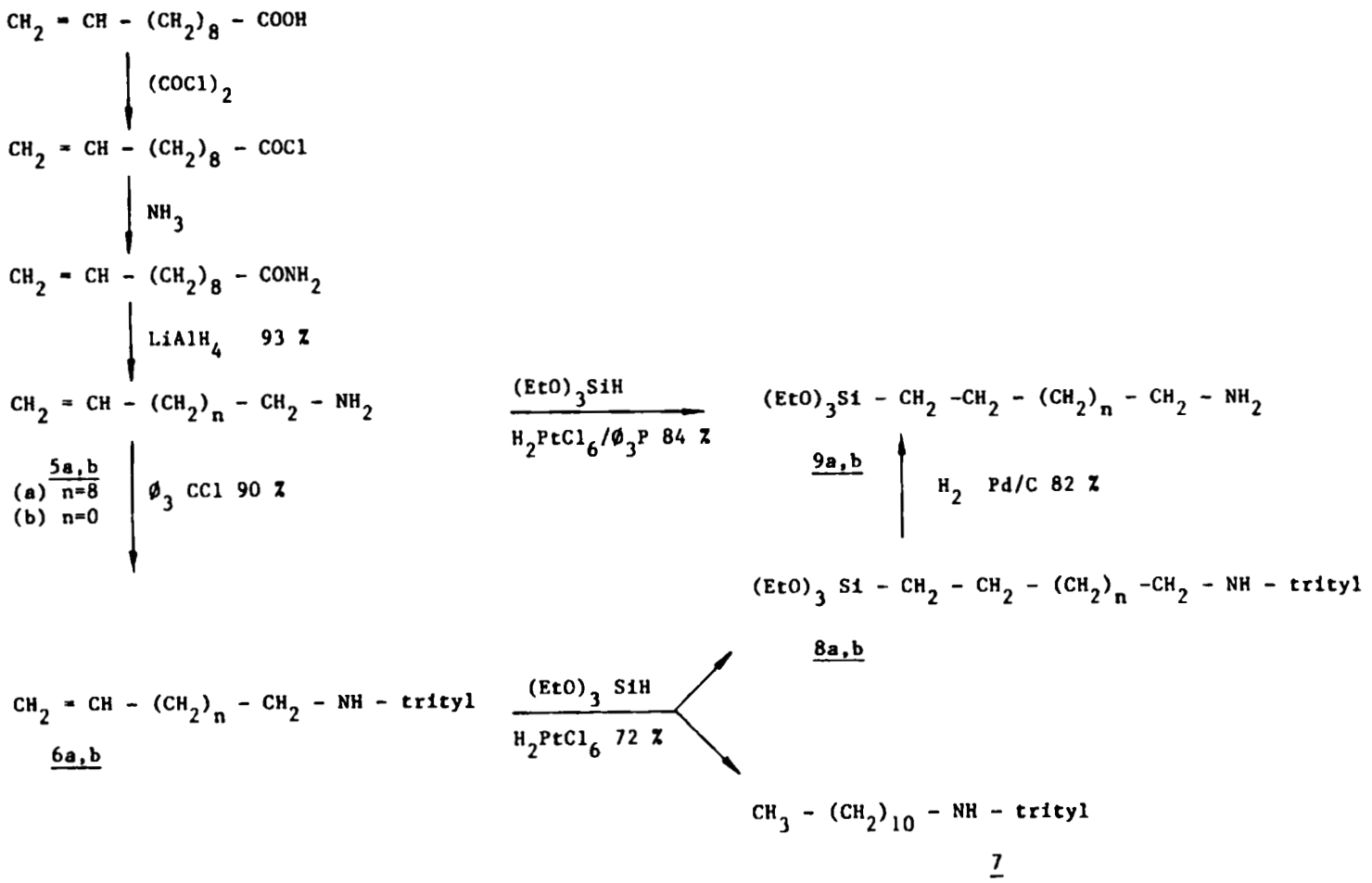

FIGURE 1

Supports functionalised with amino acids

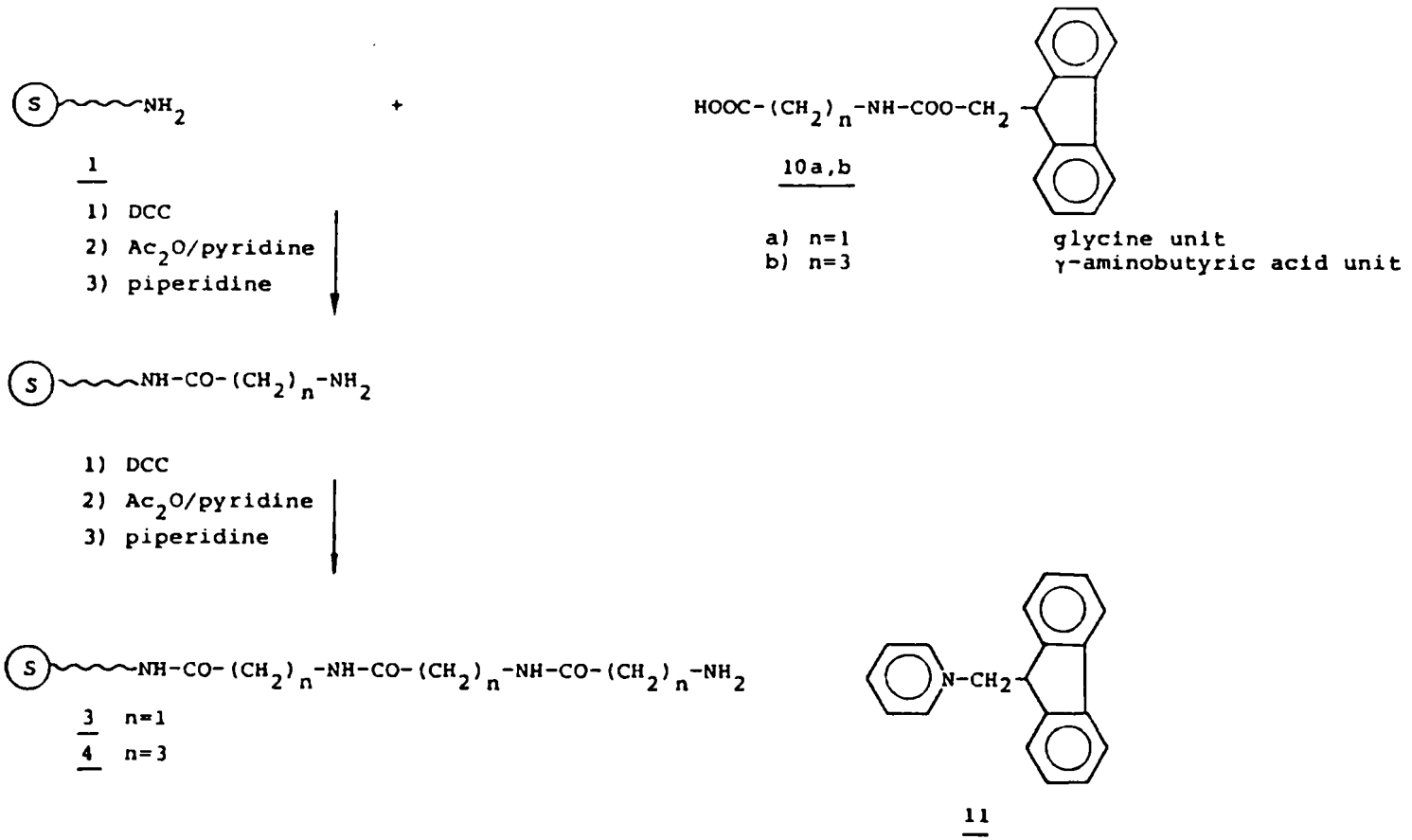

FIGURE 2

of bound spacer (see Table 2). The introduction of the second unit went faster than the first one.

Comparison of the different supports. The first nucleoside is incorporated on the different supports by reacting it with the terminal free amine function. This is done by activation of the nucleoside 3'-succinate ester with p-nitrophenol[12]. In all cases the support was loaded with deoxyguanosine and then a condensation was run, first with deoxyadenosine and second with deoxyguanosine again. This sequence seemed interesting to evaluate the different supports because condensation between two purines gave the worst results on aminopropyl silica. Similar results were also seen on a polystyrene support[13].

The assembly procedure is as follows : the support containing 4 µmol of deoxyguanosine was put in an Omnifit system, and the cycle shown in Table 3 was carried out. From the results in Table 4 one can see that aminopropyl silica with a double glycine spacer improves the yield of the first condensations almost up to the level of subsequent cycles (between 85 and 95 %). This effect was not observed with the double γ-aminobutyryl spacer. The aliphatic long chain alkylamine support incorporated the first nucleoside 100 % in contrast to the other supports, but the yields of the first condensation cycles didn't improve.

The favourable results with the double glycine spacer were confirmed during the synthesis of some 30 oligonucleotides of which a hexadecamer in 18 % overall yield (9 monomer and 3 dimer additions) (see Fig. 3).

Yields for the first two condensation steps are summarized in table 5 for 6 different oligonucleotides.

The oligo's are deprotected with 0.5 M oximate[15] (o-nitrobenzaldoxime/tetramethylguanidine) in dioxane-water 1:1 for 24 h. The support is already trityl negative after 12 h if synthesis is done on silica with a double glycine spacer in contrast with syntheses on 1, where 24 h is necessary. The eluate is run quickly over a column of Dowex 50WX8 (pyridinium form), evaporated and further deprotected with concentrated ammonia. Isolation is done on RP-HPLC[16], followed by detritylation with 80 % acetic acid.

TABLE 4. Condensation yields on the different supports.

Support		5'AG	5'GAG
Aminopropyl silica 1	(70 µmol/g)	61 %	66 %
Aminoundecyl silica 2	(68 µmol/g)	65 %	67 %
Diglycyl-aminopropyl silica 3	(50 µmol/g)	79 %	81 %
Di-(γ-aminobutyryl)-aminopropyl silica 4	(70 µmol/g)	61 %	67 %

On the four different supports the 5'GAG trimer was synthesized according to Table 3. Between brackets the loading of the first nucleoside is shown.

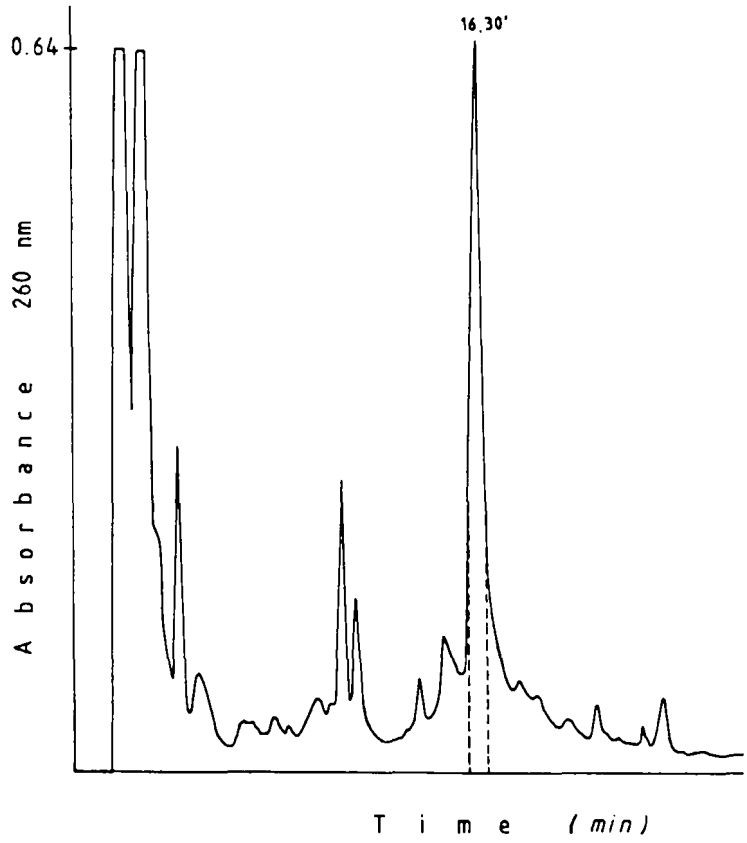

Fig. 3. Purification of a hexadecamer (CTGACTCTGAAAATTG) as its tritylated species on a µ-Bondapak C18 10 µm column (25.0 x 0.4 cm I.D.). Buffer A, 0.1 % triethylammonium acetate (TEAA), pH 7.1. Buffer B, 0.1 M TEAA, pH 7.1 in 80 % acetonitrile. Gradient, 20-50 % B in 25 min at 1.5 ml/min, ambient temperature.

TABLE 5. Yields of the first two condensation steps

A)	3'G	50 µmol/g	B)	3'G	50 µmol/g
	TG	85 %		GG	92 %
	CTG	90 %		TGG	88 %
	Further cycles	87 %		Further cycles	89 %
C)	3'A	42 µmol/g	D)	3'C	51 µmol/g
	GA	76 %		TC	77 %
	CGA	85 %		ATC	87 %
	Further cycles	86 %		Further cycles	87 %
E)	3'G	50 mol/g	F)	3'G	50 mol/g
	GG	77 %		TG	86 %
	TGG	86 %		TTG	85 %
	Further cycles	83 %		Further cycles	87 %

Condensation yields of the first two cycles are shown for some oligonucleotides. Note the difference for B and E showing the influence of circumstances, especially for manual synthesis (dryness of solvents, drying of building blocks and syringes...). Pannel F shows the results of the first two condensation steps of the hexadecamer, which HPLC profile is shown in Figure 3.

Figure 4 shows an electrophoretic autoradiogram of the same hexadecamer, after a single purification step with reverse phase chromatography. Along with it are shown two hexamers and a decamer. This last one was synthesized on silica functionalised without spacers.

Discussion of coupling yields. This work deals with functionalisation of silica by 4 different lengths of alkylamine spacers. The idea behind this work ensued from reported observations indicating that the coupling yield for the first nucleotide is significantly decreased, possibly due to steric interactions. It is clear today that more than one parameter might be operative e.g. silica loading, degree of capping of the free hydroxyl groups on the silica surface and pore size.

Therefore the different supports have been synthesized from the same batch of Fractosil 500 silica gel, excluding the influence of pore size. Capping of the final product also was done in the same way. The loading of the different supports was the same for 1, 2 and 4. Only the

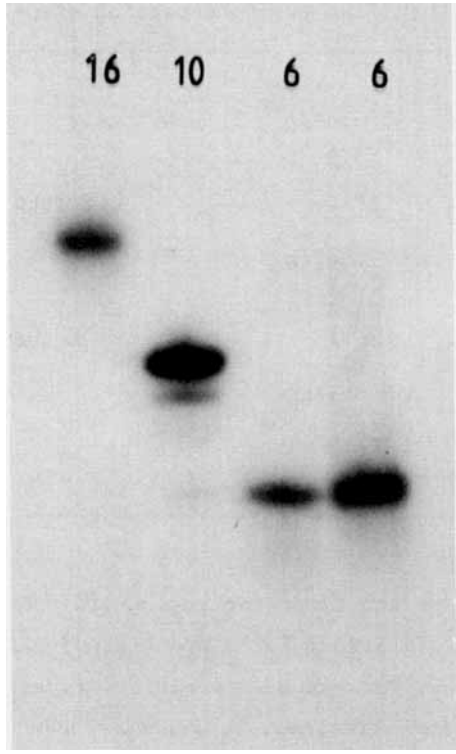

FIGURE 4

support with the double glycine unit had a slightly lower loading, but this difference is not significant enough to explain the favourable results. The diglycyl unit is also claimed to be necessary on a polydimethylacrylamide-kieselguhr support[17], but comparative yields were to our knowledge never reported with or without this spacer. Introduction of a double β-alanine spacer on a polystyrene support on the other hand didn't improve the results[18].

The double glycine unit has a repetition of short polar stretches of almost planar amide bonds. This might cause the first nucleoside to be pushed away from the apolar silica surface which is end capped. A γ-aminobutyric acid unit on the other hand might fold back easier and form intramolecular bonds causing no further distance between the apolar silica surface and the more polar nucleoside. The aliphatic long chain

alkylamine could fold back because of hydrophobic interactions and likewise would not function as a spacer causing greater distance between surface and nucleoside. The fact that this aminoalkylsilica reacts completely during the loading reaction might be because the ω-aminogroup is pushed out of the apolar layer.

Note though the difference from 77 % up to 92 % for the same GG condensation (panel B and E,Table 5). This indicates that not just the spacer is important, but that other factors do play their role, like the drying of the support and the building blocks or maybe the amount of free hydroxylgroups still uncapped on the support, which would retain some water more strongly.

All these are mere speculations as evidence for these explanations is absent. The results though show clearly an improvement with the double glycine moiety incorporated between the silica surface and the first nucleoside.

EXPERIMENTAL

Materials and Methods

Nitromethane was distilled over $CaCl_2$. Pyridine was refluxed overnight and distilled twice, once from tosylchloride and once from KOH, and kept over molecular sieves 4A. $ZnBr_2$, N-methylimidazole (kept over molecular sieves 4A), dimethylaminopyridine (DMAP), 1,3-diclohexylcarbodiimide (DCC), chlorotrimethylsilane, aminopropyl triethoxysilane, p-nitrobenzoylchloride, undecylenic acid, chlorotriphenylmethane, triethoxysilane and hydrogen hexachloroplatinate (IV) hydrate were bought from Aldrich.

A 1 % solution of the hexachloroplatinate was made in isopropyl alcohol and kept in the dark. The solution becomes orange and a black precipitate is formed under influence of light.

Fractosil 200, 500 and 1000, all 40-63 μm, were bought from Merck. Mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT) was prepared according to M. Gait[11].

Base protected nucleosides were prepared by the transient protection procedure[19], followed by monomethoxytritylation and phosphoryla-

tion with 1,9 eq. o-chlorophenylphosphodichloridate and 5,7 eq. triazole[20], except for deoxyguanosine which was phosphorylated according to V. Efimov[21]. N-protected amino acids were prepared according to Carpino and Han[22].

UV and visible measurements were done with a Beckman UV 5230 spectrophotometer. For GLC a Pye 104 was used with FID detection and a 60 ml/min stream of nitrogen as the carrier gas. The column was a OV_1 with 3 % coating (5 feet on 4 mm inner diameter). NMR Measurements were done with a Jeol FX90Q.

ω-Aminoalkyl silica (1 and 2). To a suspension of 5 g silica gel (Fractosil 500, 40-63 μm) in 20 ml EtOH (95 %) ± 12 mmol ω-aminoalkyltriethoxysilane (9a,b) is added. The mixture is shaken at room temperature for 2 h (150 rpm), filtered, washed with EtOH (95 %) and Et_2O and dried. After heating 1 h at 110°C the silica is shaken again for 2 h in 25 ml MeOH-H_2O 1:1, washed (MeOH, Et_2O) and dried at 100°C. The remaining silanol functions are capped with chlorotrimethylsilane (3 ml) in 15 ml pyridine for 2 h, with shaking at room temperature. After washing (CH_2Cl_2 - 1 % pyridine, MeOH, Et_2O) and drying (vacuum), the silica is ready for reaction with the activated nucleosides[12]. Derivatisation was successful also in boiling toluene (150°C, shaking overnight)[5].

Determination of the functionality of 11-aminoundecyl silica. A suspension of 11-aminoundecyl silica (0.5 g) and p-nitrobenzoylchloride (0,5 g) in 5 ml pyridine is shaken 1 h at 150 rpm. The silica is filtered, washed with pyridine, MeOH, Et_2O and dried. Ninhydrin test[23] is faintly positive. 100 mg of the derivatised support is hydrolyzed during 1 h with 5 ml boiling NaOH 2 N. After cooling the mixture is filtered, washed with NaOH 2 N, and the filtrate diluted to a total volume of 1,000 ml with 0.5 N NaOH. The absorption of the p-nitrobenzoate is determined at 275 nm. The results are almost the same if determined by succinylation and subsequent esterification with p-nitrophenol[9].

ω-Undecylenylamine 5a. A solution of 18.3 g (0.1 mol) undecylenic amide (prepared from the acid) in Et_2O is added dropwise to a suspension of 5 g $LiAlH_4$ in 500 ml Et_2O. The mixture is refluxed overnight, 50 ml of water is added and the upper solution is decanted. The preci-

pitate is washed twice with Et_2O. The organic layers are dried and evaporated and the undecylenylamine 5a is purified by distillation under reduced pressure ($bp_{0.1}$ 72°C, yield 92 %); IR (CH_2Cl_2): 2930, 2860, 1645 cm^{-1}; NMR ($CDCL_3$) δ : 1.07 (NH_2), 1.31 (m, 14H, $(CH_2)_7$), 1.99 (m, 2H, C:C-CH_2), 2,66 (t, 2H, J = 6.5 Hz, CH_2N), 4.76-5.08 (m, 2H, CH_2:C), 5.54-6.02 (m, 1H, CH:C); MS m/e: 169 (M^+).

N-Trityl-ω-undecylenlyamine 6a. A solution of 5.07 g (30 mmol) undecylenylamine 5a and 13.9 g (50 mmol) chlorotriphenylmethane in 100 ml pyridine is heated overnight at 100°C. After addition of 5 ml NaOH 10 N over 30 min., the mixture is filtered, evaporated and coevaporated twice with toluene. The remaining oil was taken up in petroleum ether and filtered through a short silica column. Evaporation gave a colourless oil (yield: 11.1 g, 90 %). IR (CH_2Cl_2): 3080, 3020, 2930, 2860, 1645, 1600, 1490 cm^{-1}. MS m/e : 411 (M^+). The N-trityl-allylamine is prepared in the same way but in a shorter reaction time (5b). Melting point: 85°C; IR (CH_2Cl_2): 3080, 3020, 2840, 1645, 1600, 1490 cm^{-1}; NMR ($CDCL_3$) δ: 1.84 (brs, NH) 2,96 (m, CH_2N), 5.12-5.60 (m, CH_2:C), 5.92-6.40 (m, CH:C), 7.2-7.8 (m, trityl).

N-Trityl-11-aminoundecyl-triethoxysilane 8a. A mixture of 12.33 g (30 mmol) N-trityl-ω-undecylenylamine 6a, 8.85 g (54 mmol) triethoxysilane and 0.5 ml of a 1 % solution of H_2PtCl_6.6aq. in isopropyl alcohol is left at room temperature overnight under nitrogen atmosphere, after which it is heated 4 h at 60°C. The excess triethoxysilane is removed with a nitrogen stream, and the remaining oil is purified by distillation under reduced pressure with a Kügelrohr at 230°C. Yield: 12.5 g (72.5 %). IR (CH_2Cl_2): 3080, 3020, 2930, 2860, 1600, 1490, 1390, 1165, 1100, 1080, 958 cm^{-1}; NMR ($CDCL_3$) δ : 0.60 (m, CH_2Si), 0.8-1.68 (m, $(CH_2)_9$). 1.24 (t, J = 7.1 Hz, 3 xCH_3), 2.11 (t, J = 6.5 Hz, CH_2N), 3.76 (q, J = 7.1 Hz, 3 x CH_2OSi), 7.0-7.56 (m, trityl). MS m/e: 5.75 (M^+). The reaction was monitored with GLC (column temperature between 250°C and 300°C) and TLC (benzene-acetone 98:2, R_f 0.52).

The N-trityl-3-aminopropyl-triethoxysilane was prepared by heating the appropriate mixture for 2 h at 85°C. Distillation with a Kügelrohr at 170°C, GLC: column temperature 220°C, yield: 90 %; IR (CH_2Cl_2): 3080, 3020, 2960, 2920, 2885, 1600, 1490, 1390, 1165, 1100, 1080, 958

cm^{-1}; NMR ($CDCL_3$) δ : 0.60 (m, CH_2Si), 1.20 (t, J = 7.1 Hz, 3 x CH_3), 1.56 (m, CH_2CH_2Si), 2.08 (m, CH_2N), 3.77 (q, J = 7.1 Hz, 3 xCH_2OSi), 7.0-7.56 (m, trityl).

11-Aminoundecyl-triethoxysilane 9a.

Method A. A solution of 8.625 g (15 mmol) N-trityl-11-aminoundecyl-triethoxysilane 8a in 150 ml EtOH is hydrogenated for 4 h at 350 kPa in the presence of 1 g $Pd(OH)_2/C$. The mixture is filtered, evaporated and taken up in MeOH. Column chromatography with 400 g XAD-2 and elution with MeOH gave 4.5 g (13.5 mmol, 90 % yield) 9a as a colourless, clear fluid. IR (CH_2Cl_2) : 3410, 2920, 2860, 1390, 1170, 1100, 1080, 960 cm^{-1}; NMR ($CDCl_3$) δ : 0.62 (m, CH_2Si), 1.22 (t, J = 7.1 Hz, 3 x CH_3), 1.27 (m, $(CH_2)_9$), 2.73 (m, CH_2N), 3.81 (q, J = 7.1 Hz, 3 x CH_2OSi), 5.5 (brs, NH_2). MS m/e: 333 (M^+).

Method B. A mixture of 7.27 g (43 mmol) undecylenylamine 5a, 12.6 g (77 mmol) triethoxysilane, 0.22 ml of a 1 % solution of H_2PtCl_6.6aq. in isopropyl alcohol is heated 4 h at 130°C. Excess triethoxysilane is removed with a nitrogen stream and the remaining oil is purified by distillation under reduced pressure with a Kügelrohr at 200°C. Monitoring of the reaction with GLC (column temperature 200°C). Yield: 11,984 g (83.7 %). After removal of the excess triethoxysilane the oil can be used as such for reaction with silica gel.

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