Journal of Chromatography, 397 (1987) 13–24
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1107

SYNTHESIS AND IMMOBILIZATION OF A NOVEL ACRIDINE DERIVATIVE ON MICROPARTICULATE SILICA

A STUDY OF ITS INTERACTIONS WITH SINGLE-STRANDED OLIGONUCLEOTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

RAINER BISCHOFF*,* and FRED E. REGNIER
Purdue University, Department of Biochemistry, West Lafayette, IN 47907 (U.S.A.)

SUMMARY

A novel approach for immobilizing acridine on 5-μm silica gel is described. The acridine moiety is functionalized with a carboxylic acid group at its reactive 9-position and activated, leading to 9-acridinylpropionic acid N-hydroxysuccinimide ester. This derivative is efficiently bound to the silica matrix through a primary aliphatic amine group at the end of a fifteen-atom spacer arm. The chromatographic properties of the final stationary phases, as evaluated with $d(T)_{10}$ and $d(A)_{10}$ at various pH values and organic solvent concentrations, resemble those of hydrophobic weak anion exchangers. When a secondary amine group is placed close to the acridine moiety in one of the packings, enhanced binding of the oligodeoxyribonucleotides is observed that goes beyond a purely additive effect.

INTRODUCTION

Various acridine derivatives have been known for almost a century because of their pharmaceutical properties[1]. Especially the discovery of their value as antimalaria agents prompted the chemical synthesis of a wide variety of derivatives in the 1930s[2]. Their mode of action remained unclear until 1961 when Lerman[3] formulated the intercalation model for the interaction between acridines and double-stranded nucleic acids, which was later refined by Waring[4]. This model postulated that the planar heterotricyclic aromatic ring system of an acridine moiety slides in between two adjacent base pairs to form a very tight complex with the nucleic acid. Intercalation accounted for the mutagenic properties of some of the acridine drugs, since it disturbed the correct base pairing between the two strands of genomic DNA[5,6]. Another interesting feature of the interaction between acridines and nucleic acids by intercalation was that it caused a partial unwinding of negatively supercoiled DNA[4]. This resulted in a higher affinity of acridines towards negatively supercoiled plasmid

* Present address: Transgene S.A., 11 Rue de Molsheim, 67082 Strasbourg Cédex, France.

0021-9673/87/$03.50

DNA compared to nicked or relaxed forms. One method for isolating supercoiled plasmid DNA was therefore based on density-gradient centrifugation of a crude plasmid preparation in the presence of ethidium bromide, an intercalating acridine derivative[7]. In another application, covalently closed circular DNA from simian virus 40 (SV 40), having different numbers of superhelical turns (topoisomers), was fractionated by gel electrophoresis in an agarose gel containing ethidium bromide[8].

The strong affinity of acridines towards nucleic acids with their selectivity for double-stranded and especially covalently closed circular DNA prompted a number of investigators to immobilize acridine derivatives on solid supports with the goal of using their specificity in chromatographic separations of oligonucleotides and nucleic acids. Bünemann and Müller[9] immobilized chemically synthesized acrylamide derivatives of intercalating molecules by polymerizing them onto the surface of polyacrylamide beads. This resulted in supports that were able partially to resolve DNA, due to its GC-*versus*-AT base-pair content, depending on the base-pair specificity of the immobilized dye. A similar support was used with acridine yellow as the intercalating dye. This packing material was successfully employed in the isolation of covalently closed circular DNA[10]. In another approach, the intercalating dye acriflavin was immobilized on epichlorohydrin-activated agarose as described by Egly and Porath[11]. The column material was suitable for the separation of oligonucleotides, but clear evidence that intercalation occurred was not given[12,13]. This might be due to the fact that immobilization was performed through a primary aromatic amine group that was part of the intercalating ring system.

In this paper we describe the chemical synthesis of an acridine derivative and its subsequent immobilization on microparticulate silica through a fifteen-atom spacer arm. Two types of stationary phases were prepared, one that contained the acridine moieties as the only ionic binding sites and another one that had an equivalent amount of secondary amines as additional sites for ionic interactions with nucleic acids. These column materials were examined at different pH values and organic solvent concentrations, in order to evaluate their ionic and hydrophobic properties.

EXPERIMENTAL

Chemicals

9-Chloroacridine was purchased from Eastman Kodak (Rochester, NY, U.S.A.). $d(T)_{10}$ and $d(A)_{10}$ were obtained from Pharmacia (Piscataway, NJ, U.S.A.). Kieselgel 60 F_{254} thin-layer chromatography plates were a product of E. Merck (Darmstadt, F.R.G.) and obtained through EM Science (Cherry Hill, NJ, U.S.A). Vydac 101TBP 5.5-μm (330-Å) silica was a gift from The Separations Group (Hesperia, CA, U.S.A.). All other chemicals were of the highest purity available from either Mallinckrodt (Paris, KY, U.S.A.) or Aldrich (Milwaukee, WI, U.S.A.).

Instrumentation

^{1}H NMR spectra were recorded on a T60A (60-MHz) or a FT 80 (80-MHz) instrument from Varian Assoc., Instrument Group (Palo Alto, CA, U.S.A.). High-performance liquid chromatography (HPLC) was performed with a Vista 5500 chromatograph (Varian), equipped with a Spectroflow 773 variable-wavelength detector (Kratos Analytical Instruments, Westwood, NJ, U.S.A.) at a flow-rate of 0.5 ml/min.

Elemental analysis was performed by the Microanalysis Laboratory of Purdue University, Department of Chemistry.

Synthesis of 9-acridinylpropionic acid N-hydroxysuccinimide ester (I)

9-Acridinylpropionic acid was synthesized essentially as previously described[14].

9-Methylacridine. 9-Chloroacridine (25 g, 117 mmol) was allowed to react with freshly distilled diethyl malonate (27.5 ml, 182 mmol) and sodium (4.2 g, 182 mmol) in 50% dry toluene and 50% absolute ethanol. To start the reaction, 9-chloroacridine was first heated in dry toluene until refluxing occurred. The hot suspension was then added to the already refluxing solution of diethyl malonate and sodium in absolute ethanol and refluxed for 16 h. After adding 230 ml of 50% (v/v) aqueous hydrochloric acid, the organic solvents were carefully distilled off, and the mixture was refluxed for another 4 h. Finally, 230 ml water were added and the hot solution was filtered through charcoal. Upon cooling, 9-methylacridinium hydrochloride deposited slowly from the solution. It was filtered off and heated in 575 ml water for 2 h. Undissolved material was removed by filtration of the hot mixture, and 9-methylacridine was precipitated with about 60 ml 2 *M* (aqueous) sodium hydroxide and filtered off. After drying the material for 8 h at 50°C under vacuum, 11.4 g (59 mmol) of crude product were obtained. The dried pellet was refluxed in 250 ml ethyl acetate and filtered. Evaporation of the ethyl acetate gave 9.25 g (48 mmol, 41%) of 9-methylacridine, m.p. 118–119°C. Its purity was determined by thin-layer chromatography (TLC) on silica gel (R_F = 0.28, 2% methanol in dichloromethane) and 60-MHz ^{1}H NMR spectroscopy in C^2HCl_3: δ (relative to tetramethylsilane, TMS) 8.23–7.93 (m, 4 H); 7.80–7.15 (m, 4 H); 2.97 (s, 3 H).

9-Bromomethylacridine. 9-Methylacridine (9.25 g, 48 mmol) and N-bromosuccinimide (8.52 g, 48 mmol) were dissolved in 180 ml tetrachloromethane. To start the reaction, benzoyl peroxide (0.47 g, 1.9 mmol) was added, and the solution was refluxed for 4.5 h. The hot mixture was filtered quickly, and the precipitate that formed upon cooling was recrystallized from 150 ml tetrachloromethane, giving 7.55 g of 9-bromomethylacridine (27.8 mmol, 58%). The product appeared to be pure, as determined by TLC on silica gel (R_F = 0.50, 2% methanol in dichloromethane) and 60-MHz ^{1}H NMR spectroscopy in C^2HCl_3: δ (relative to TMS) 9.07–8.63 (m, 4 H); 8.50–8.00 (m, 4 H); 5.73 (s, 2 H).

9-Acridinylpropionic acid. 9-Bromomethylacridine (7.55 g, 28 mmol) was added slowly to a stirred solution of freshly distilled diethyl malonate (8.5 ml, 56 mmol) and sodium (1.28 g, 56 mmol) in refluxing absolute ethanol. After refluxing the reaction mixture for 1 h, 110 ml of 50% (v/v) aqueous hydrochloric acid were added and the ethanol was distilled off. This mixture was refluxed for 4 h, cooled in ice and filtered (*ca.* 12 g air-dried material). The precipitate was dissolved in *ca.* 250 ml hot, dilute hydrochloric acid (10 ml of conc. hydrochloric acid in 1 l of water), and the pH of the solution was adjusted to 5.5 with an aqueous solution of potassium carbonate (20 g in 100 ml water). The precipitate was filtered off after cooling the solution on ice (*ca.* 8 g air-dried material) and recrystallized from glacial acetic acid (a small amount of undissolved material was filtered off after refluxing in 350 ml acetic acid). For effective crystallization, it was necessary to reduce the volume of the acetic acid by about 50 ml. Crystallization after 2 days at 4°C gave 1.25 g 9-acridinium-

propionic acid acetate (4 mmol, 14.5%). Its purity was determined by TLC on silica gel (R_F = 0.53, *n*-butanol–acetic acid–water, 8:1:1) and elemental analysis (found: C 70.6, H 5.1, N 4.7%, calculated: C 69.5, H 5.5, N 4.5%).

9-Acridinylpropionic acid N-hydroxysuccinimide ester (I). 9-Acridiniumpropionic acid acetate (1 g, 3.3 mmol) was suspended in 170 ml dichloromethane and activated by the addition of 1,1'-carbonyldiimidazole (1.33 g, 8.3 mmol) for 1 h at 20°C. N-Hydroxysuccinimide (0.8 g, 8.3 mmol) was added and the reaction mixture was stirred overnight at 20°C, after which the starting material was completely dissolved. The solution was extracted three times with 670 ml 0.1 *M* sodium dihydrogenphosphate (pH 6) containing 0.5 *M* sodium chloride and the organic layer was dried over sodium sulphate. For crystallization of the product, *n*-hexane was added to the dichloromethane phase until it turned slightly cloudy (*ca.* 150 ml *n*-hexane). The product crystallized at −20°C (*ca.* 3 days), forming long, yellow needles. It was filtered off, dried and stored in a vacuum desiccator over calcium chloride. Yield: 0.73 g (65.3%) (R_F = 0.56 on silica gel, 10% methanol in dichloromethane). It was characterized by elemental analysis (found: C 68.7, H 4.5, N 7.9%, calculated: C 69.4, H 4.6, N 8.1%) and 80-MHz ^{1}H NMR spectroscopy in C^2HCl_3: δ (relative to TMS) 8.42–8.20 (m, 4 H); 8.00–7.53 (m, 4 H); 4.10 (t, 2 H, CH_2-acridine); 3.07 (t, 2 H, CH_2COON); 2.92 (s, 4 H, $OCCH_2CH_2CO$); $J(CH_2–CH_2)$ = 9 Hz.

Synthesis of stationary phases

A 5-g amount of microparticulate silica (Vydac®, 5.5 μm, pore diameter 330 Å) was suspended in 50 ml dry toluene, containing 250 μl dry diisopropylethylamine (DIPEA), and 5 ml glycidoxypropyltrimethoxysilane were added. After refluxing the suspension overnight, the solid was filtered off, washed with dimethylformamide, then methanol and dried.

The glycidoxypropyl-derivatized silica (5 g) was suspended in 20% triethylamine in dichloromethane (125 ml), containing 11.6 g 1,6-diaminohexane (100 mmol). The reaction proceeded at 20°C overnight with gentle shaking. The solid was filtered off washed with dichloromethane, then methanol and dried. The ionic binding capacity was determined by picric acid analysis of 10 mg of the material as previously described[15]. For the following reactions the packing was divided into four batches of 1 g each.

Acridine-Support I. Silica support (1 g), containing about 60 μmol of 1,6-diaminohexane (1,6-DAH-Support), was suspended in 10 ml dichloromethane containing 28 μl (0.2 mmol) triethylamine and allowed to react overnight with 55 mg (0.16 mmol) of compound I at 20°C with gentle shaking. The solid was filtered off, washed with dichloromethane, methanol, diethyl ether and dried. Its ionic binding capacity was determined by picric acid analysis.

The support was resuspended in 10 ml dichloromethane, containing 35 μl (0.2 mmol) DIPEA and allowed to react overnight with 25 mg (0.16 mmol) acetic acid N-hydroxysuccinimide ester at 20°C with gentle shaking. Afterwards, the material was filtered off washed and its ionic binding capacity determined, as described above.

Control-Support I. Control-Support I was synthesized by a procedure analogous to that for the Acridine-Support I where instead of compound I an equivalent amount of acetic acid N-hydroxysuccinimide ester was employed.

Acridine-Support II. A 500-mg amount of Acridine-Support I was allowed to

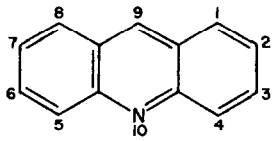

Fig. 1. Chemical formula of the acridine ring system.

react overnight with 500 μl (5.3 mmol) acetic anhydride and 650 mg (5.3 mmol) 4-dimethylaminopyridine (DMAP) in 10 ml dichloromethane, containing 1.75 ml (10 mmol) DIPEA, at 20°C with gentle shaking. After filtration and washing of the packing, its ionic binding capacity was determined by picric acid analysis.

Control-Support II. A 500-mg amount of the 1,6-DAH-Support was allowed to react with acetic anhydride as described for the preparation of Acridine-Support II.

RESULTS

Immobilization of acridine derivatives on different kind of supports has been performed with the goal of separating nucleic acids on the basis of the ability of the

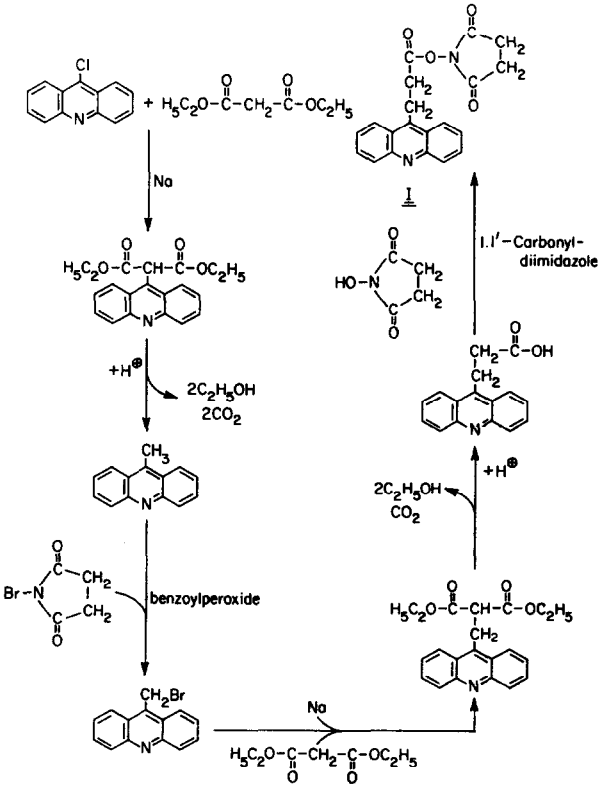

Fig. 2. Reaction scheme for the synthesis of 9-acridinylpropionic acid N-hydroxysuccinimide ester (I).

dye to intercalate between adjacent base pairs. The procedures described started with either polyacrylamide[9] or agarose[11] as matrices, and one of the published procedures[11] employed a functional group that was part of the intercalating ring system.

In this paper, we first describe the chemical synthesis of 9-acridinylpropionic acid N-hydroxysuccinimide ester (I), a derivative that could readily be immobilized on primary aliphatic amine-containing stationary phases in high yield. This compound has a couple of advantageous features. First, the reactive functional group is attached to the acridine ring through the 9-position, thus preventing interference with its intercalating properties (for numbering of the ring system see Fig. 1). Secondly, electron-withdrawing substituents at the 9-position of the acridine ring system render the bond to the already electron-deficient carbon atom more susceptible to hydrolysis. Therefore, the electron-withdrawing carboxylic acid ester was separated from the acridine moiety by two methylene groups (see compound I in Fig. 2). This added to

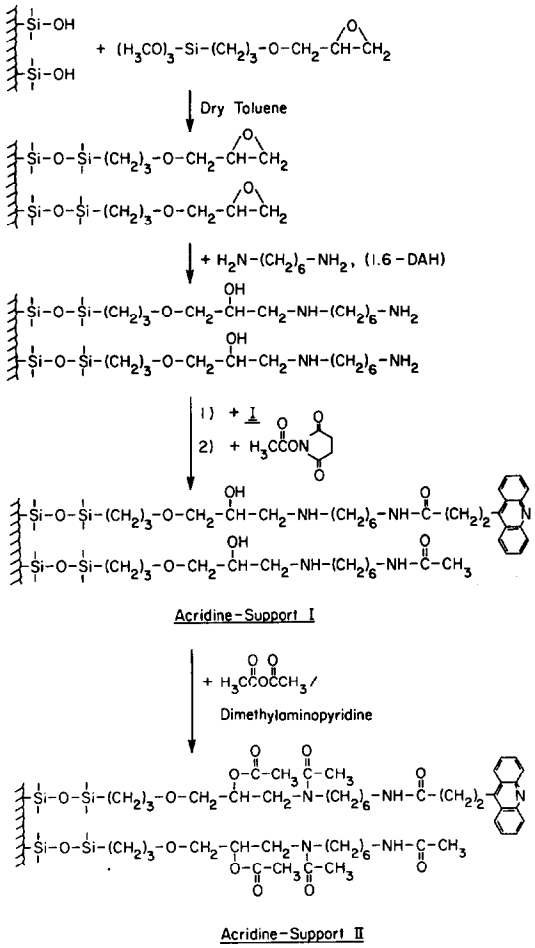

Fig. 3. Reaction scheme for immobilizing 9-acridinylpropionic acid N-hydroxysuccinimide ester on silica gel via a spacer arm.

the stability of the final packing materials, which have been used for about 8 months without any detectable loss in binding capacity. Thirdly, the N-hydroxysuccinimide ester used to activate the carboxylic acid functionality on the acridine moiety is highly specific for primary aliphatic amine groups bound to supports. Thus, it is possible to immobilize selectively the acridine molecules at the end of a long spacer arm ending in a primary amine (see Fig. 3).

The synthesis of compound I involved a number of well established organic synthetic reactions mostly based on diethyl malonate chemistry (see Fig. 2). However, attention had to be paid to two special problems in the first reaction. First, 9-chloroacridine is very moisture sensitive, and secondly, it is not totally soluble in the solvent mixture used. Thus in order to obtain a high yield, it was important to assure good stirring and to prevent any moisture from reaching the reaction mixture. The bromination of 9-methylacridine proceeded efficiently, since the 9-methyl group is activated by the electron-deficient carbon atom at the 9-position of the acridine ring system. The conversion of 9-bromomethylacridine into 9-acridinylpropionic acid was straightforward. However, the final isolation of the product proved to be very tedious, since the zwitterionic compound was difficult to crystallize, owing to its limited solubility. Crystallization from glacial acetic acid gave a pure product in low yield (14.5%). Since the amount isolated was sufficient to perform the final activation reaction and the preparation of the stationary phases, an optimization of the isolation procedure was not attempted. The synthesis of the activated N-hydroxysuccinimide ester of 9-acridinylpropionic acid I (see Fig. 2) gave a pure product after recrystallization from dichloromethane–*n*-hexane. 1,1'-Carbonyldiimidazole as a coupling agent gave higher yields than dicyclohexylcarbodiimide and was therefore preferred. Its use also facilitated the isolation of the final product, since its breakdown products are water soluble and could be removed by aqueous extractions. Compound I appeared to be stable when stored in a vacuum desiccator over calcium chloride. Because of its good solubility in organic solvents, such as dichloromethane, it could easily be employed to acylate immobilized primary aliphatic amine groups with high efficiency.

In designing the packings, two features were taken into account in order to enhance their selectivity. First, the acridine moiety was immobilized through a long, hydrophilic spacer arm. Secondly, it was assured that the acridines were the primary binding sites for DNA on the packings, *i.e.*, binding to the spacer arm or the silica gel itself was kept to a minimum.

Fig. 3 outlines the synthesis of the packings. In the first step, the silica surface was functionalized with reactive epoxide moieties by derivatizing it with glycidoxypropyltrimethoxysilane in dry toluene. The epoxy residues were then allowed to react further with an excess of 1,6-diaminohexane to introduce a spacer arm between the primary amine groups and the silica surface. This was the central support (1,6-DAH-Support) for synthesizing the acridine-containing stationary phases (Acridine-Supports I and II, see Fig. 3) as well as the Control Supports, where the acridine moieties were replaced by acetyl groups. Its ionic binding capacity was determined to be 120 μmol/g by a static binding assay with picric acid, as previously described[15]. Since each 1,6-diaminohexane moiety was able to bind two picric acid molecules, this meant that 60 μmol of 1,6-diaminohexane were bound per g of silica. The rather low ligand density of about 0.75 μmol/m^2 surface area (surface area = 80 m^2/g, as determined

by the manufacturer) reflects the number of epoxy groups that were able to react with 1,6-diaminohexane. However, from the elemental carbon analysis, a ligand density of 2,4 μmol epoxy groups/m² was calculated. This indicates that a large number of epoxy groups were either not reactive towards 1,6-diaminohexane, or more likely had already been hydrolyzed by the silica surface. Since the affinity of DNA towards acridine is relatively high, we did not attempt to increase the ligand density, as this might lead to difficulties in recovering the DNA from the column.

In the next step, the primary amine groups of the 1,6-DAH-Support were acylated with compound I. No change in ionic binding capacity was observed after the acylation reaction, because each acridine moiety bound a picric acid molecule through its tertiary amine ring nitrogen. In order to determine the amount of immobilized acridines, the residual free primary and secondary amine groups were acetylated with acetic anhydride (Acridine-Support II in Fig. 3). The remaining ionic binding capacity of 77 μmol/g was due to 65.5 μmol/g acridine moieties and 11.5 μmol/g secondary amine groups that were inaccessible to acetylation with acetic anhydride. The amount of free secondary amines was determined independently by reacting the amine groups of the 1,6-DAH-Support with acetic anhydride and measuring the residual ionic binding capacity (Control-Support II in Table I). Some of the 1,6-DAH-Support was also treated with acetic acid N-hydroxysuccinimide ester to give Control-Support I with an ionic binding capacity of about 50%, compared to the original matrix (see Table I). This result indicated that the active ester reacted selectively with the primary amine groups. It was also shown that no further reduction in ionic binding capacity was observed when the 1,6-DAH-Support was first made to react with compound I and then with acetic acid N-hydroxysuccinimide ester (Acridine-Support I in Table I and Fig. 3). This result confirmed that compound I acylated all the primary amine groups but left the secondary amines free. Therefore, it was safe to assume that the acridine moieties were immobilized through the primary amine groups, and full use of the fifteen-atom spacer arm was made.

The synthetic procedures described led to one packing where the acridine moieties were the only ionic binding sites (Acridine-Support II) and another one where their binding was assisted by an equivalent amount of secondary amines (Acridine-Support I). These stationary phases were complemented by Control-Supports, containing acetyl groups instead of acridine moieties. Thus, it was possible in the following chromatographic evaluations to differentiate between the effect of the acridine

TABLE I

IONIC BINDING CAPACITY (μmol/g) OF THE SYNTHESIZED SUPPORTS DETERMINED BY PICRIC ACID ANALYSIS[15]

Control-Supports contain acetyl groups instead of acridine moieties.

	Total capacity	*Primary amines*	*Secondary amines*	*Acridines*
1,6-DAH-Support	120	60	60	–
Acridine-Support I	120	–	56	64
Acridine-Support II	77	–	11.5	65.5
Control-Support I	56	–	56	–
Control-Support II	11.5	–	11.5	–

functionalities relative to the contribution of the spacer arm and the silica matrix to retention of oligonucleotides.

The chromatographic properties of the synthesized matrices were investigated at various pH values and organic solvent concentration in the mobile phase. While the pH dependence of the capacity factor, k', revealed the weak anion-exchange character of the supports, the influence of the organic solvent concentration on k' gave insight into their hydrophobic properties[16]. Two oligodeoxyribonucleotides, $d(T)_{10}$, containing a weakly hydrophobic nucleobase, and $d(A)_{10}$, with a stronger hydrophobic character, were used to examine the stationary phases. Both Acridine-Supports showed a decreasing affinity for the oligonucleotides with increasing pH in the mobile phase (see Fig. 4). This result reflected the decreasing number of ionic binding sites with increasing pH, since the amount of protonated amines decreased. When chromatographing the oligonucleotides under identical mobile phase conditions on the corresponding Control-Support, which lack the acridine moieties, no retention was observed. Therefore, it was concluded that the pH dependence of k' was mainly due to deprotonation of the tertiary amine nitrogen in the acridine ring system, which has a pK_a value of about 6 (determined by titration). However, an enhanced affinity of both oligonucleotides for the Acridine-Support I, where the secondary amine group in the spacer arm is free, compared to Acridine-Support II, was an indication that the secondary amine was participating in retention. The necessity of using a higher sodium chloride concentration in the mobile phase to elute the oligomers isocratically from Acridine-Support I was particularly pronounced in the case of $d(A)_{10}$. This, together with the result that no retention of either $d(T)_{10}$ or $d(A)_{10}$ occurred on Control-Support I, where the secondary amine was the only ionic binding site, led to the conclusion that there was a synergistic effect of enhanced oligonucleotide binding to Acridine-Support I between the acridine moieties and the secondary amines. Similar effects have been observed for oligonucleotides and nucleic acids on anion-exchange matrices, where hydrophobic and ionic binding sites were in close proximity (mixed-mode packings)[16–19]. A contribution of hydrophobic interactions to the retention of $d(T)_{10}$ and $d(A)_{10}$ on the Acridine-Supports was indicated by the difference in their k' values at pH 7, depending on the sodium chloride concentration (see Fig. 4). When $d(A)_{10}$ was eluted with a higher concentration of

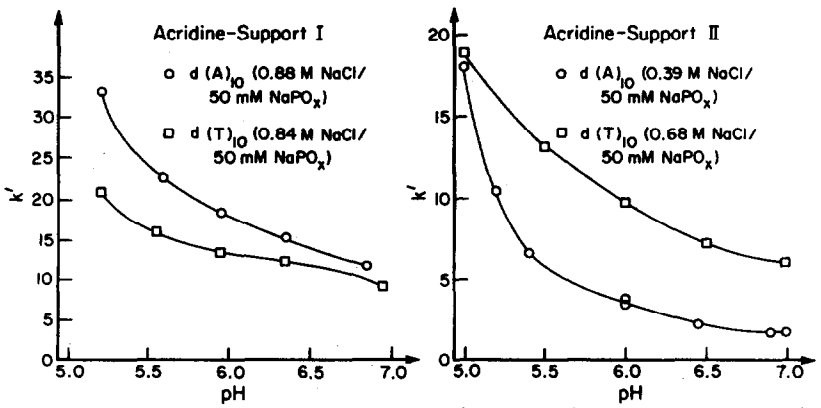

Fig. 4. Dependence of the capacity factor, k', of $d(T)_{10}$ and $d(A)_{10}$ on the pH in the mobile phase. $NaPO_x$ stands for sodium phosphate.

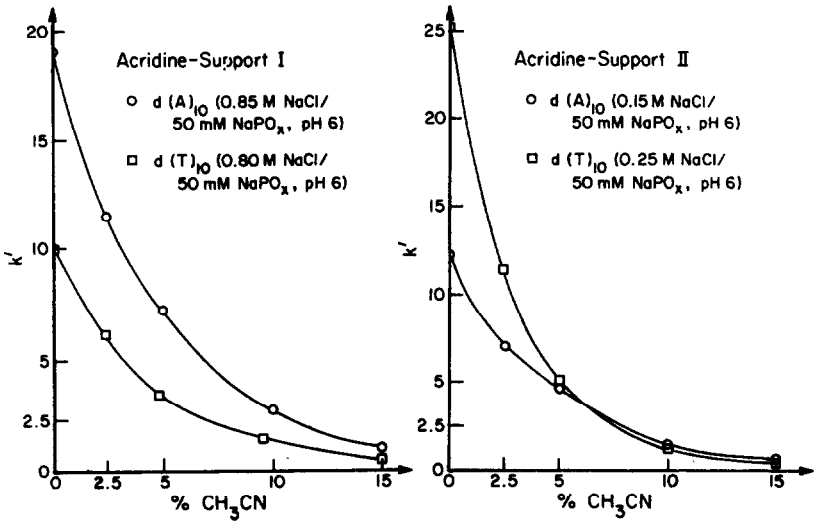

Fig. 5. Dependence of the capacity factor, k', of $d(T)_{10}$ and $d(A)_{10}$ on the acetonitrile concentration in the mobile phase. $NaPO_x$ stands for sodium phosphate.

sodium chloride in the mobile phase than $d(T)_{10}$, it had a larger k' value at pH 7 and *vice versa*. This led to the assumption that the increased salt concentration enhanced hydrophobic interactions with the immobilized acridine moieties.

In order to evaluate the hydrophobic properties of the packing materials, $d(T)_{10}$ and $d(A)_{10}$ were chromatographed isocratically at pH 6 with increasing amounts of acetonitrile in the mobile phase. On both Acridine-Supports a pronounced decrease in the k' values was observed when acetonitrile was added to the buffer (see Fig. 5). This indicated that the stationary phases interacted hydrophobically with the oligonucleotides. These hydrophobic interactions could be ascribed to the bound acridine moieties, since the corresponding Control-Supports did not retain the oligomers. The effect of the acetonitrile concentration on the k' values was most pronounced below 5%. By adding 15% acetonitrile to the mobile phase the k' values for $d(T)_{10}$ and $d(A)_{10}$ dropped almost to zero on Acridine-Support I (free secondary amine groups), while only 10% acetonitrile was needed on Acridine-Support II (acetylated secondary amines) to achieve a similar decrease.

The results presented showed that both Acridine-Supports functioned like hydrophobic weak anion exchangers when evaluated with single-stranded oligonucleotides. Retention could be influenced by either changing the pH value, thus affecting the amount of protonation of the ionic binding sites, or by varying the organic solvent content of the mobile phase with a pronounced effect on the hydrophobic interactions.

DISCUSSION

In this publication we describe the chemical synthesis of 9-acridinylpropionic acid N-hydroxysuccinimide ester (I), an acridine derivative that was immobilized on

microparticulate silica containing primary aliphatic amine groups, with high efficacy. The derivative had to fulfill the following conditions: (a) the intercalating properties of the acridine ring system should not be reduced; (b) the acridine moiety should be immobilized through a bond that is hydrolytically stable under chromatographic conditions and (c) the derivative should be stable upon long time storage but highly reactive under the conditions of the immobilization reaction.

The first condition was met by derivatizing the acridine ring system through its electrophilic 9-position, since it was known that such derivatives still exhibited intercalating properties. The activated ester functionality was separated from the 9-position by two methylene groups, in order to achieve a hydrolytically stable linkage. As indicated by the low field shift of the ^{1}H NMR signals for the methyl group in 9-methylacridine (δ 2.97) and especially for the methylene protons in 9-bromomethylacridine (δ 5.73), there was a strong electron-withdrawing effect from the 9-position of the acridine system, which is enhanced by other electronegative substituents, such as bromine atoms. Therefore, it was concluded that another electron-withdrawing group like a carboxylic acid amide at the methylene carbon atom next to the 9-position might render this bond rather susceptible to hydrolysis upon prolonged exposure to aqueous media, thus limiting the lifetime of the supports. Activation of the carboxylic acid group via its N-hydroxysuccinimide ester was chosen because of its high reactivity and selectivity towards primary aliphatic amine groups as well as its stability when stored under dry conditions. While all of the synthetic steps leading to compound I gave yields of 40–60%, synthesis of 9-acridinylpropionic acid yielded only 15% product. This low yield was partly due to incomplete recovery during the work-up. It seems that recrystallization from glacial acetic acid did not proceed with good recovery. We did not investigate this problem further, since the amounts isolated were sufficient for performing the final reaction step and for preparing the stationary phases.

Derivatization of the silica gel was performed so that a rather hydrophilic matrix was obtained, containing a long spacer arm ending in a primary aliphatic amine group. This was achieved by first derivatizing the silica matrix with glycidoxypropyltrimethoxysilane. The comparatively low ligand density of primary amines (0.75 μmol/m^2) on the final packing indicated a partial loss of epoxide groups after the first reaction. This loss might be due to hydrolysis of epoxy groups catalyzed by the acidic nature of the silica surface, since the carbon content, as determined by elemental analysis, indicated a significantly higher ligand density. However, the strong affinity of nucleic acids to acridines seemed to favor low ligand-density packings. Therefore, no attempt to increase the ligand density was made. Another important feature of the packings was the hydrophilicity of the spacer arm. This was indicated by almost identical retention times of $d(T)_{10}$ and $d(A)_{10}$ when chromatographed with a gradient of increasing sodium chloride concentration on Control-Support I, since oligodeoxyadenylic acids are known to be more strongly retained than the corresponding oligodeoxythymidylic acids on anion-exchange matrices exhibiting some hydrophobic properties[16,18]. Control-Support I contains a spacer arm with acetylated primary and free secondary amine groups, making it a good candidate for determining the properties of the spacer.

The determination of the ionic binding capacity by picric acid analysis, which measures the primary and secondary amines in the spacer arm as well as the basic

acridine moieties in combination with selective acetylation of primary amines by acetic acid N-hydroxysuccinimide ester, and the complete acetylation of all accessible secondary and primary amines by acetic anhydride, gave an accurate measure of the amount of immobilized acridine moieties and of the free secondary amines left in the spacer arm (see Table I). This was important in terms of understanding how acridines on their own or in combination with adjacent secondary amines interact with nucleic acids. The results indicate that there was a synergistic enhancement of binding of single-stranded oligonucleotides to acridine moieties by the free secondary amine groups. The enhancement occurred at a salt concentration which was well above the one where retention by the secondary amines alone occurred. This result correlated well with previous observations that two or more functional groups in close proximity to each other were able to interact with the solutes simultaneously, enhancing retention beyond a purely additive effect. The Acridine-Supports retained oligonucleotides in a way reminiscent of earlier described mixed-mode packings, where hydrophobic and ionic binding sites were in close proximity.

We are presently investigating the properties of the packing materials in relation to double-stranded and negatively supercoiled DNA. This should lead to a better understanding of the interaction of acridines with nucleic acids under various conditions in a defined environment. It is especially interesting to find out whether the free secondary amine group in the spacer arm will improve the intercalating properties of the acridine moiety, an effect that was observed by others[20].

ACKNOWLEDGEMENTS

This work was supported by grant GM 29144 from the National Institutes of Health. This is paper No. 10975 from the Purdue Agricultural Experiment Station.

REFERENCES

1 A. Bernthsen, *Liebigs Ann. Chem.*, 224 (1884) 1.
2 O. J. Magidson and A. M. Grigorowski, *Chem. Ber.*, 69 (1936) 396.
3 L. S. Lerman, *J. Mol. Biol.*, 3 (1961) 18.
4 M. J. Waring, *J. Mol. Biol.*, 54 (1970) 247.
5 L. S. Lerman, *J. Cell Physiol.*, 64 Sup. 1 (1964) 1.
6 M. J. Waring, *Annu. Rev. Biochem.*, 50 (1981) 159.
7 R. Rabloff, W. Bauer and J. Winograd, *Proc. Natl. Acad. Sci. U.S.A.*, 57 (1967) 1514.
8 W. Keller, *Proc. Natl. Acad. Sci. U.S.A.*, 72 (1975) 4876.
9 H. Bünemann and W. Müller, *Nucleic Acids Res.*, 5 (1978) 1059.
10 W. S. Vincent III and E. S. Goldstein, *Anal. Biochem.*, 110 (1981) 123.
11 J.-M. Egly and J. Porath, *J. Chromatogr.*, 168 (1979) 35.
12 J.-M. Egly, *J. Chromatogr.*, 215 (1981) 243.
13 E. Boschetti, P. Girot, A. Staub and J.-M. Egly, *FEBS Lett.*, 139 (1982) 193.
14 A. Campbell, C. S. Franklin, E. N. Morgan and D. J. Tivey, *J. Chem. Soc.*, (1958) 1145.
15 A. J. Alpert and F. E. Regnier, *J. Chromatogr.*, 185 (1979) 375.
16 R. Bischoff and L. W. McLaughlin, *J. Chromatogr.*, 270 (1983) 117.
17 R. Bischoff, E. Graeser and L. W. McLaughlin, *J. Chromatogr.*, 257 (1983) 305.
18 R. Bischoff and L. W. McLaughlin, *J. Chromatogr.*, 296 (1984) 329.
19 R. Bischoff and L. W. McLaughlin, *J. Chromatogr.*, 317 (1984) 251.
20 P. Laugâa, Z. Markovits, A. Delbarre, J.-B. Le Pecq and B. P. Roques, *Biochemistry*, 24 (1985) 5567.