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3-(2,4,5,7-TETRANITROFLUORENIMINO)-PROPYLDIETHOXYSILOXANE —A HIGHLY SELECTIVE, BONDED π -COMPLEXING PHASE FOR HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A bonded, charge-transfer type stationary phase packing for high-pressure liquid chromatography has been developed. The bonded phase is stable under various solvent conditions and affords very high selectivity for alkyl-substituted polynuclear aromatic hydrocarbons. Both the capacity ratio and the plate height are strong functions of the stoichiometric phase mass. A satisfactory compromise is achieved by reducing the stoichiometric charge-transfer phase mass (moles/g) and secondary adsorption effects were minimized by the synthesis, *in situ*, of a "mixed" bonded phase.

INTRODUCTION

A wide variety of packings is now available for high-pressure (or high-speed) liquid chromatography (HPLC). In general, these packings can be divided into three general classes, *viz.* adsorptive, coated liquid phase and "bonded" or "brush" type phases. The first and last types offer the greatest practical advantages since solvent programming is possible and the need to use saturator precolumns is avoided. Bonded phases offer the possibility of "tailoring" an adsorbent for a given separation although the exact nature of bonded phases is still in question¹. The bonded phases reported to date are generally of the class type, that is, polar or non-polar in terms of the classes of compounds exhibiting selective retention. For example, consider the series octadecylsilyl, phenyldimethylsilyl, and cyanodimethylsilyl silica gel. Bonding involves a chemical reaction with the surface hydroxyl groups of silica gel to form C-O-Si or Si-O-Si bonds, the latter being more stable to varying chromatographic conditions. In addition, such species as allyl- or aminosilyl-bonded phases have been developed for hydrogen bonding or olefinic class separations.

It would seem that the really great advantage of HPLC over gas chromatography is the ability to generate non-ideal interactions in the mobile phase and to introduce mobile phase components which compete with solute molecules in selective stationary phase interactions. Several examples already exist for attempts to generate

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high selectivity within classes. Grushka and Scott² have studied bonded dipeptides in terms of their utility as selective phases for amino acids. A particularly fertile area would seem to be carefully designed charge-transfer reagents. Charge-transfer interactions have been shown to be extremely solvent dependent³ and the strength of the charge-transfer interaction should be readily moderated by appropriate solvent programming. Two recent studies have considered the utility of charge-transfer in HPLC. Mikes *et al.*⁴ have reported excellent separations and, in addition, compared formation constants obtained by gas-liquid chromatography (GLC). The formation constants observed in HPLC were, in some cases, 20–40 times those obtained in GLC and, in addition, the values of relative retention (α) were greater, thereby enhancing separation. Karger *et al.*⁵ have reported on liquid-solid experiments involving aromatic hydrocarbon adsorbates and a siliceous support coated with a classical GLC additive and π -complexing reagent, 2,4,7-trinitrofluorenone. Although the study was complicated by the high formation constants observed in terms of high resistance-to-mass-transfer and consequent peak broadening, these authors demonstrated that this liquid-solid system was of substantial analytical utility for ring-positional isomers of polynuclear aromatics.

Interest in polynuclear aromatics arises from their potential carcinogenicity. Alkyl derivatives of polynuclear hydrocarbons are often more potent carcinogens than the non-substituted analogs (*e.g.*, methyl cholanthrene and cholanthrene, methyl benzanthracene and benzanthracene). In this paper, we report the successful synthesis and use of a bonded charge-transfer phase for HPLC specifically designed for high selectivity for positional isomers of alkyl-substituted polynuclear hydrocarbons. Resistance-to-mass-transfer is observed in "heavily-loaded" columns but is overcome by stoichiometric control and the use of a "mixed", bonded stationary phase system.

EXPERIMENTAL

A Molecular Separations (Champion, Pa., U.S.A.) pulseless, liquid chromatograph was used for all measurements. Inlet pressure was monitored on a 3000-p.s.i. gauge graduated in 25-p.s.i. increments (Heise, Newtown, Conn., U.S.A.). Eluting solutes were monitored by a low-volume ultraviolet detector operating at 254 nm. Sample injection was achieved with a 1- μ l high-pressure sampling valve (Valco Instruments, Houston, Texas, U.S.A.). Columns were precision-bore 0.250-in.-O.D. \times 0.084-in.-I.D. stainless steel (Superior Tube, Norristown, Pa., U.S.A.). Packing of the carefully cleaned columns was achieved by the balanced-density slurry method at 6000 p.s.i.⁶.

The basic support for the experiments was a microporous silica gel of 10- μ m particle diameter and 500 m²/g surface area (SI 60 "Lichrosorb"; E. Merck, Elmsford, N.Y., U.S.A.). Alkylation and stoichiometric amination were achieved by modification of established procedures^{7,8} utilizing 3-aminopropyltriethoxysilane and methyltriethoxysilane. Amine activity (mequiv./g) of the phase prior to the formation of the imine charge-transfer group was determined by non-aqueous titrimetry. In addition, total wt. % carbon and total wt. % nitrogen were determined by classical methods. Packings were exhaustively extracted (with refluxing acetone and then with refluxing acetonitrile) in a Soxhlet apparatus prior to use.

All solvents were reagent grade and used without further purification. Solutes were recrystallized by appropriate methods prior to use. In critical cases, structure was confirmed by spectroscopic measurements.

RESULTS AND DISCUSSION

The title stationary phase was prepared by means of the two-step synthesis given in Fig. 1. In the initial step, the amine function was introduced to the surface of Lichrosorb via exhaustive reaction of surface silanols with ethoxy groups of 3-aminopropyltriethoxysilane. This amine phase (amine activity, 1.09 mequiv./g by titration) was reacted with 2,4,5,7-tetranitro-9-fluorenone (TENF) giving the tetranitrofluorenimine phase. Partial evidence for a successful reaction could be seen in the color change which occurred. The aminated silica gel was white but the imine packing closely resembled graphitized carbon black. The dark color was not the result of the reaction conditions, which were quite mild, but was more reasonably attributable to the introduction of the $-N=C$ chromophore. The selection of the TENF group was based on the following considerations: (1) it was believed that a TENF imine might be less subject to hydrolysis, (2) tetranitrofluorenes form stronger charge-transfer complexes than the tri- or dinitro-analogs, which might increase resistance-to-mass-transfer but, if properly moderated, could enhance selectivity, and (3) symmetrical nitration of the fluorene renders the interactions between solute and solvent (donor and acceptor) statistically equivalent (this would not be true in a trinitrofluorene, for example) and enhances the possibility of selective steric interaction.

The "heavily loaded" column (25 cm) exhibited excessively large capacity factors for polyaromatic solutes larger than anthracene or fluoranthene (see Fig. 2). In a hydrocarbon solvent strong tailing, indicative of apparent poor mass transport kinetics⁵, was evident. Nevertheless, the large α values were encouraging. In an effort to determine whether reduction of the total charge-transfer phase mass would yield useful retention times, a short (8 cm) column was packed and evaluated. Results with a more polar solvent, acetonitrile, appeared to indicate that the system could have

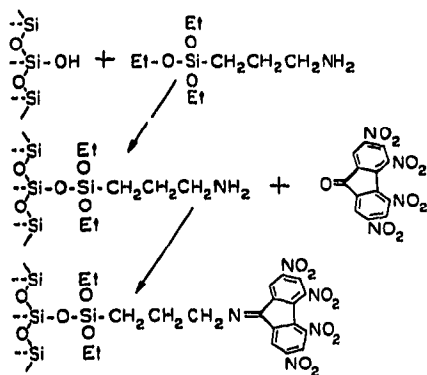


Fig. 1. Two-step synthesis of 3-(2,4,5,7-tetranitrofluorenimino)-propyldiethoxysiloxane.

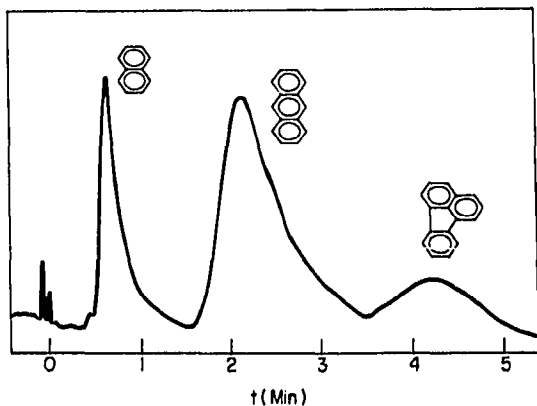


Fig. 2. Chromatogram of naphthalene, anthracene and fluoranthene on a heavily loaded column. Inlet pressure, 1200 p.s.i.; flow-rate, 1.2 ml/min.

analytical utility if the effective per cent "liquid loading" of charge-transfer phase could be reduced approximately tenfold (the capacity factor, k' , of 7-methylbenz[*a*]-anthracene = 50-100).

Several possibilities exist for achieving a net smaller mass of bonded charge-transfer phase. First, the charge-transfer packing could be diluted with unreacted silica gel to give one-tenth the charge-transfer phase mass per unit weight of packing. This is objectionable since untreated silica gel might introduce a second sorption isotherm to the separation process. Secondly, dilution could be achieved by mixing a deactivated silica gel (trimethylsilylated?) with the charge-transfer packing. Thirdly, a mixed bonded phase could be generated with tenfold less amine activity than the original packing. The second possibility was rejected on the basis that truly homogeneous mixing of the silica packings would be difficult to achieve. The third route was selected.

After extensive experimentation, a chemically bonded amine phase was prepared with one-seventh the original activity (0.16 mequiv./g by titration). This phase was produced by first exhaustively reacting the surface with an excess of methyltriethoxysilane in refluxing distilled water adjusted to pH 3 with glacial acetic acid followed by a reaction with a controlled amount of the amine coupling reagent in refluxing dry methanol. Such a procedure was utilized because the kinetics of the amine coupling reaction are highly favorable⁷ and may even involve the displacement of the methyl deactivating reagent by the amine. The amine phase was reacted with TENF to produce a reduced-activity fluorenimine stationary phase [0.26 mequiv./g of fluorenimine by calculation from elemental analysis; analytical: C = 6.25%, N = 1.82%; theoretical: C = 5.25% (from amine excluding methylation reaction products) N = 1.82%]. Although one can only speculate about the possibility of large-scale polymer formation on the support, evidence from a series of reactions does not lend much support to such a conclusion. Even under extreme conditions (twentyfold excess of methyl coupling reagent, 24-h reaction in 0.1 *M* acetic acid under reflux) only 5.74% carbon was present in the elemental analysis, corresponding to 0.96 mequiv./g of the methyl coupling reagent or essentially monolayer coverage on the support. It is of

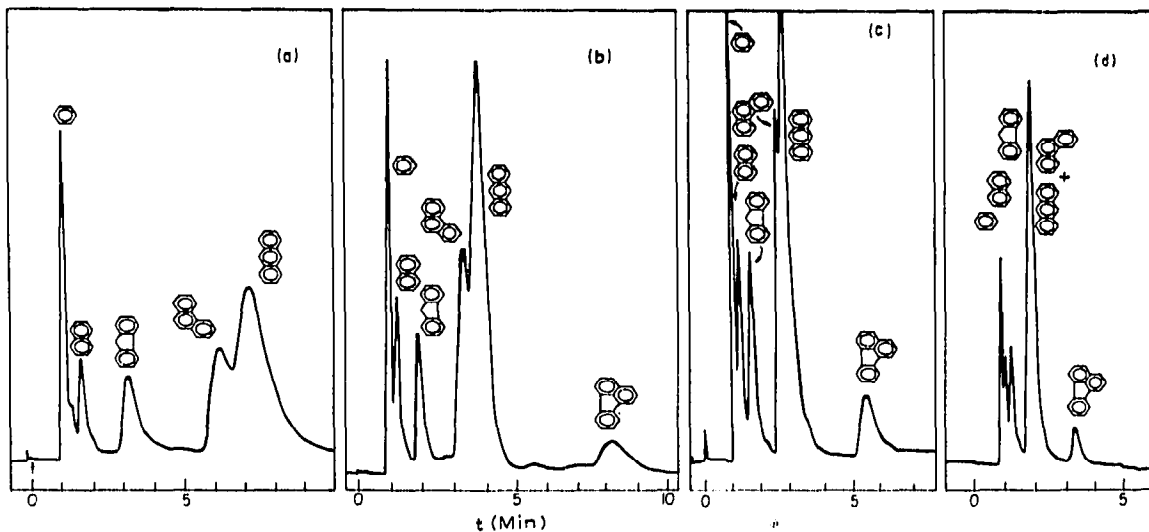


Fig. 3. Chromatograms illustrating the effect of solvent on retention, selectivity and efficiency. Solvents: (a) isooctane, (b) 87.5% isooctane-12.5% acetonitrile-saturated isooctane, (c) 50%-50%, (d) 100% acetonitrile-saturated isooctane. Inlet pressure, 1200 p.s.i.; flow-rate, 1.2-1.3 ml/min.

course possible that "polymer patches" as suggested by Grushka and Kikta⁹ might be present on the support surface and such a possibility may be worth further investigation.

The results of experiments with columns prepared with the lower charge-transfer phase mass packing are shown in Figs. 3 and 4 and Tables I-III. All k'

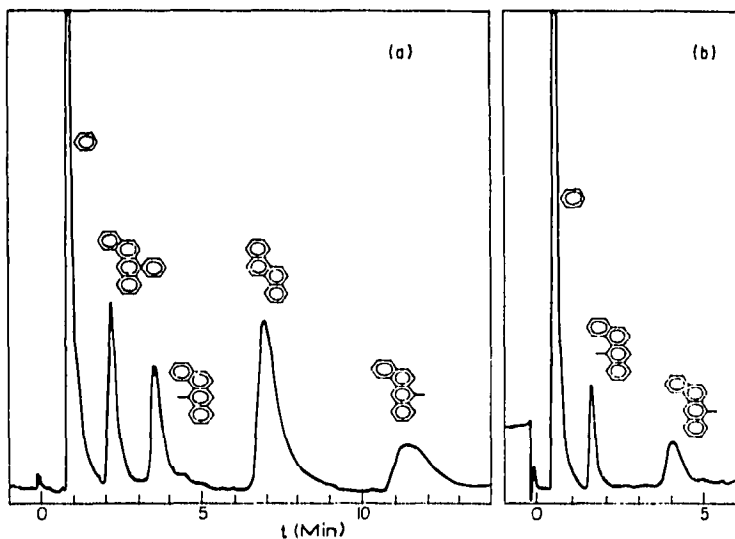


Fig. 4. Representative chromatograms for larger aromatic hydrocarbons. Solvents: (a) acetonitrile-saturated isooctane, (b) pure acetonitrile.

TABLE I

VARIATION OF RETENTION TIME (t_R , min) AND CAPACITY FACTOR (k') OF VARIOUS SOLUTES WITH SOLVENT COMPOSITION

ASI = Acetonitrile-saturated isooctane; I = isooctane.

Solute	100% ASI- 0% I		50% ASI- 50% I		25% ASI- 75% I		12½% ASI- 87½% I		0% ASI- 100% I	
	t_R	k'	t_R	k'	t_R	k'	t_R	k'	t_R	k'
Benzene	0.93		0.91		0.81		0.87		0.93	
Naphthalene	1.08	0.17	1.16	0.28	1.13	0.40	1.31	0.50	1.42	0.52
Fluorene	1.31	0.41	1.58	0.74	1.67	1.08	1.62	0.85	2.74	1.94
Phenanthrene	1.89	1.03	2.45	1.70	2.76	2.42	3.27	2.75	5.38	4.77
Anthracene	1.89	1.03	2.70	1.97	3.04	2.77	3.65	3.18	6.43	5.90

TABLE II

RETENTION TIMES (t_R , min) AND CAPACITY FACTORS (k') FOR BENZ[*a*]ANTHRACENE AND CHRYSENE DERIVATIVES

ASI = acetonitrile-saturated isooctane; I = isooctane.

Solute	100% ASI-0% I		50% ASI-50% I	
	t_R	k'	t_R	k'
7-Phenylbenz[<i>a</i>]anthracene	2.23	1.41	5.00	4.51
12-Methylbenz[<i>a</i>]anthracene	3.61	2.89	7.18	6.91
Chrysene	7.01	6.55	11.96	12.2
6-Methylchrysene	7.01	6.55	15.39	15.9
7-Methylbenz[<i>a</i>]anthracene	11.68	11.6	24.32	25.8

TABLE III

RETENTION TIMES (t_R , min) AND CAPACITY FACTORS (k') FOR SOME LARGER POLYNUCLEAR AROMATIC HYDROCARBONS

Solvent, acetonitrile.

Solute	t_R	k'
Pyrene	1.7	1.13
Tetracene	1.8	1.25
Chrysene	3.0	2.75
Benzo[<i>a</i>]pyrene	8.0	9.0
1-Methylcholanthrene	2.8	2.50
2-Methylcholanthrene	3.9	3.88
3-Methylcholanthrene	14.7	17.4

values were calculated assuming benzene to be essentially unretained and were corrected, where applicable, for retention due to the methyltriethoxysilyl component of the mixed phase. This contribution was estimated from experiments with a fully methyltriethoxysilylated packing and assuming the retention mechanisms of the two components to be additive. The contribution of the alkyl phase was quite small, as can be seen by inspection of the chromatograms shown in Fig. 5. The solvent system used in all other studies was isooctane-acetonitrile, which has the advantage that both

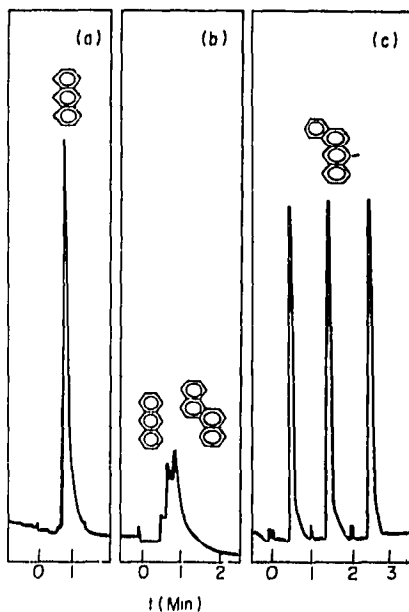


Fig. 5. Chromatograms obtained using exhaustively methyldiethoxysilylated silica gel packing: (a) anthracene; (b) anthracene and chrysene; (c) three consecutive injections of 7-methylbenz[*a*]anthracene. Solvent, isooctane; flow-rate, 1.2 ml/min; inlet pressure, 1200 p.s.i.

solvents exhibit a low ultraviolet cut-off and have practically the same viscosity. The latter permits solvent programming at constant pressure and constant flow-rate. In addition, acetonitrile was thought to be a potentially strong competitor for the charge-transfer process in the column, thereby enhancing the effect of solvent composition. In point of fact, it was observed that in isooctane carrier, acetonitrile and fluoranthene have essentially the same k' value.

One of the better examples of selectivity in the hydrocarbon series studied thus far is shown in the large difference in relative retention values observed for 12-methyl- vs. 7-methylbenz[*a*]anthracene in all the solvents studied (Table II). The exact reason for this high selectivity is not immediately evident (except the obvious fact that the formation constants must be quite different). Inspection of space-filling models for the molecular complex 2,4,5,7-tetranitrofluorenone-methylbenz[*a*]anthracene (attempting to maximize orbital overlap) indicates a strong steric interaction between the 12-CH₃ of the donor and the 4- and 5-nitro groups of the acceptor absent in the 7-CH₃ case. The methyl groups of both 7- and 12-methylbenz[*a*]anthracene are subject to strong peri-hydrogen interactions which, in the case of 12-methylbenz[*a*]anthracene in particular, may actually force the methyl group out of the plane of the aromatic ring. Similar peri-hydrogen interaction at the 7 position may account for the short retention (small k') observed for the phenylbenz[*a*]anthracene. The phenyl group should be on the average in a plane almost perpendicular to the benz[*a*]anthracene itself thus increasing steric repulsion and reducing nearness of approach. Alternatively, the difference in selectivity may be due to inductive activation of the ring by alkyl substitution which increases the donor capacity or ring electron density. Evidence for this can be seen in

the enhanced retention of 6-methylchrysene over its parent hydrocarbon chrysene, where the same models do not indicate a significant steric interaction difference.

Further evidence for the high selectivity of this type of phase is seen in the relative retention values of 1-, 2-, and 3-methylcholanthrene, given in Table III, where in the 1- and 2-methyl cases, the alkyl substituents are actually one carbon remote from the benz[*a*]anthracene backbone. The hydrocarbons elute in numerical order, with the 3-methyl derivative having much stronger retention. Of equal interest is the relative elution order of several different four- and five-ring hydrocarbons. Pyrene and tetracene are rapidly eluted by acetonitrile followed by chrysene and much later by benzo[*a*]pyrene. As might be expected, a general trend is observed in which retention increases with the number of aromatic rings. Among hydrocarbons with four or more rings it appears that the presence of an open coil of three rings in the molecule (as in phenanthrene) causes stronger retention than an analogous straight chain (as in anthracene) or an analogous closed coil (as in pyrene). Although this conclusion is based upon a relatively small number of solutes, it does not seem unreasonable in that the charge-transfer interaction likely involves a planar overlap between the slightly coiled nitrofluorenimine and the various hydrocarbons studied.

It became evident as the studies progressed that some loss of efficiency had occurred with the introduction of the chemically bonded fluorenimine stationary phase on the surface of Lichrosorb. The 10- μm Lichrosorb was originally chosen as a support for the substrate because of the well-known efficiency of such small particles in liquid chromatography. Indeed, as shown in Fig. 6, the plate height, H , for nitrobenzene eluted on a 25-cm column slurry packed with 10- μm Lichrosorb was smaller than 0.1 mm at low flow velocities and increased only slightly at higher flow velocities. In contrast, the plate height increased at a much faster rate for anthracene and pyrene eluted on the 25-cm mixed-phase fluorenimine column packed under the same conditions. Evidently, the increased slope represents an increase in resistance-to-mass-

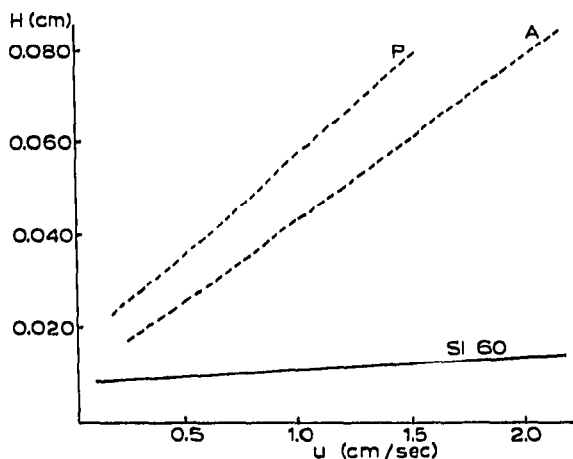


Fig. 6. Behavior of height equivalent to a theoretical plate as a function of flow velocity. Curves A and P denote the behavior of anthracene and pyrene, respectively, on a 14% TENF-imine stationary phase with acetonitrile-saturated isooctane as the solvent; SI 60 denotes the behavior of nitrobenzene on an SI 60 10- μm Lichrosorb column with water-saturated hexane as the solvent.

transfer caused by less rapid exchange of solutes from the stationary phase to the mobile phase. One possible explanation for slow solute desorption from the stationary phase is that the rates of formation and dissociation of the charge-transfer complex may be much slower than the rates for formation and dissociation of a physically adsorbed species on silica gel. It would thus seem reasonable that as the strength of the charge-transfer complex increases (from anthracene to pyrene) the exchange between the two chromatographic phases might become slower with a consequent increase in the resistance-to-mass-transfer term. An alternative explanation might be based upon the assumption that polymer networks of stationary phase have been built up on the surface, trapping some of the mobile phase into stagnant pools. While such pools might cause slow diffusion into and out of the mobile liquid phase, no evidence as yet confirms the presence of such a polymer network. It might be noted, in addition, that the loss in efficiency for the mixed fluoremine phase is minimal at low flow velocities and at higher flow velocities gives lower plate heights than typical published values for 40- μm porous-layer beads¹⁰. In addition, the high selectivity of this phase more than compensated for the small loss of efficiency in most cases.

Further work is needed to elucidate and expand the utility of this type of phase. Studies are presently underway on other fluorene-type packings with different degrees of nitration and different substituents (*e.g.*, bromine), on the effect of alkyl chain-length and of substituent bulk α to the imine group on the chain. In addition, we are studying the utility of these packings for aromatic amines and nitrogen-containing heterocycles. Spectroscopic studies of the donors, acceptors and donor-acceptor complexes and their relation to chromatographic behavior are planned.

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