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Jonathan L. Sessler[a]; John W. Genge[a]; Vladimir Král[a]; Brent L. Iverson[a]
[a] Department of Chemistry and Biochemistry, The University of Texas at Austin, Austin, Texas

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Separation of Mono-, Di-, and Triphosphate Nucleotides by Cytosine Substituted, Silica-Bound Sapphyrin Solid Supports

JONATHAN L. SESSLER*, JOHN W. GENGE, VLADIMIR KRÁL and BRENT L. IVERSON

Department of Chemistry and Biochemistry, The University of Texas at Austin, Austin, Texas, 78712

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A cytosine-substituted sapphyrin when used as a silica gel bound HPLC solid support effectively separates guanosine 5′-mono-, di-, and triphosphate from a mixture of the mono-, di- and triphosphates of cytidine, uridine, adenosine, and guanosine, respectively under isocratic conditions at pH 7. In addition, using this same solid support, cytidine 5′-monophosphate, guanosine 5′-monophosphate, adenosine 5′-monophosphate and uridine 5′-monophosphate are all readily separated from each other under similar HPLC conditions.

INTRODUCTION

Nucleotide phosphates represent an important class of compounds in biological systems.[1] Adenosine 5′-monophosphate, adenosine 5′-diphosphate, adenosine 5′-triphosphate, guanosine 5′-monophosphate and guanosine 5′-triphosphate, for example, all play critical roles in glucose metabolism (e.g., citric acid cycle and glycolysis).[2] At present, there exist few commercially available HPLC solid supports that will effectively separate mono-, di-, and triphosphate nucleotides at physiological pH.[3] Because of problems associated with charge and hydrogen binding, traditional HPLC methods for these ionic species remain limited in their applicability.[4] On the other hand, in a more general sense, efforts to effect the HPLC separation of other ionic species by silica bound macrocycles have often met with considerable success. For instance, Cram[5] and others[6] have used crown ethers and aza crown ethers to effect the separation of cationic species. Similarly, Meyerhoff *et al.* have recently reported the anion separating properties of silica bound In (III), and Sn (IV) substituted tetraphenyl porphyrins.[7]

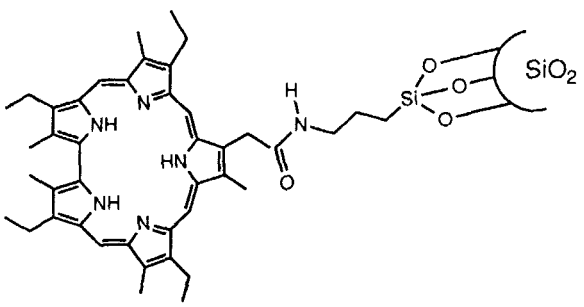
Our own efforts have focused on using sapphyrin-substituted silica gels as HPLC solid supports. Sapphyrins, a class of pentapyrrolic expanded porphyrins first reported by Woodward,[8] bind anions at neutral pH with varying degrees of affinity[9] and, when bound to HPLC grade silica gel (to give system **1**), can be used to separate effectively mono-, di-, and trinucleotides at neutral pH in a buffered solution.[10] Un-

*Corresponding author.

fortunately, these first generation silica gels were unable to separate individual nucleotides, such as CMP and GMP, from each other. In view of this, we considered preparing yet more sophisticated silica gels, derived from nucleobase-bearing sapphyrin entities. These, it was thought, would allow for the tailored separation of a chosen mononucleotide from a generalized nucleotide mixture. The validity of this supposition is illustrated in this report via the use of a cytosine-substituted sapphyrin; this latter system, when appended to silica gel allows, for the specific separation of guanine containing nucleotides from those derived from other nucleic acid bases.

The use of nucleoside-type molecules to ensure specificity in binding has been employed by many groups including our own,[11,12] as well as those of Lehn,[13] Hamilton[14] and others.[15] In the case of sapphyrin, attaching a nucleobase allows for the preferential transport of the complementary nucleotide through a separating "membrane".[11] Presumably, this transport, and the recognition phenomenon on which it is based, reflects two underlying binding modes. The first involves phosphate anion chelation by the protonated sapphyrin whereas the second derives from complementary hydrogen interactions between the nucleotides. While it is known that the latter interactions are greatly decreased in water, it was nonetheless thought that the multiple recognition-type opportunities with which each nucleotide would be faced on passing through the nucleobase-appended, sapphyrin-modified, silica gel column might still afford good separations. In the case of the cytosine-sapphyrin systems reported here, it was thus specifically posited that good separations would be achieved between guanine- and, say, cytosine- or adenine-derived nucleotides. This indeed proved to be the case.

The cytosine substituted sapphyrin needed to prepare the HPLC-grade silica gel alluded to above (system **2**), was prepared as shown in Scheme 1 (Figure 1). The column was commercially packed and tested and the number of theoretical plates (8841) determined.[16] Separations were then carried out under isocratic conditions using a 200 mM sodium phosphate buffer as the eluent. The findings from these experiments are now detailed below.



SCHEME 1

As implied by the introductory remarks, the main scientific question engendering the synthesis of the cytosine-sapphyrin-silica gel (**2**) was whether such supports could be used to effect the separation of GMP, GDP, and GTP from a mixture of X(M, D, or T)P (X = A, U, G, C). Towards this end, a 1 mM aqueous mixture of X(M, D, or T)P (X = A, U, G, C) was made up and a 20 μl sample was injected onto a 3.2 mm × 100 mm HPLC cytosine-sapphyrin silica gel column as well as on to a sapphyrin substituted silica gel "control" column that did not contain cytosine. The samples were eluted isocratically-using a 50 mM sodium phosphate buffer; pH = 7.1. In the case of the control column, as expected,[10] separation between the mono-, di-, and triphosphates (XMP, XDP, and XTP; X = A, G, C, U) is achieved but no separation is effected with respect to the different nucleotides present (Figure 3). On the other hand, when the cytosine-sapphyrin silica gel column was used, clean separation between GMP, GDP and GTP and the remaining nucleotides XMP, XDP, XTP (X = A, G, C, U) is observed (Figure 2).

Fortuitously, with the new cytosine-sapphyrin support, we not only observed the sought-for separation of guanine nucleotides from other nucleic acids (*vide supra*), we also found, in the

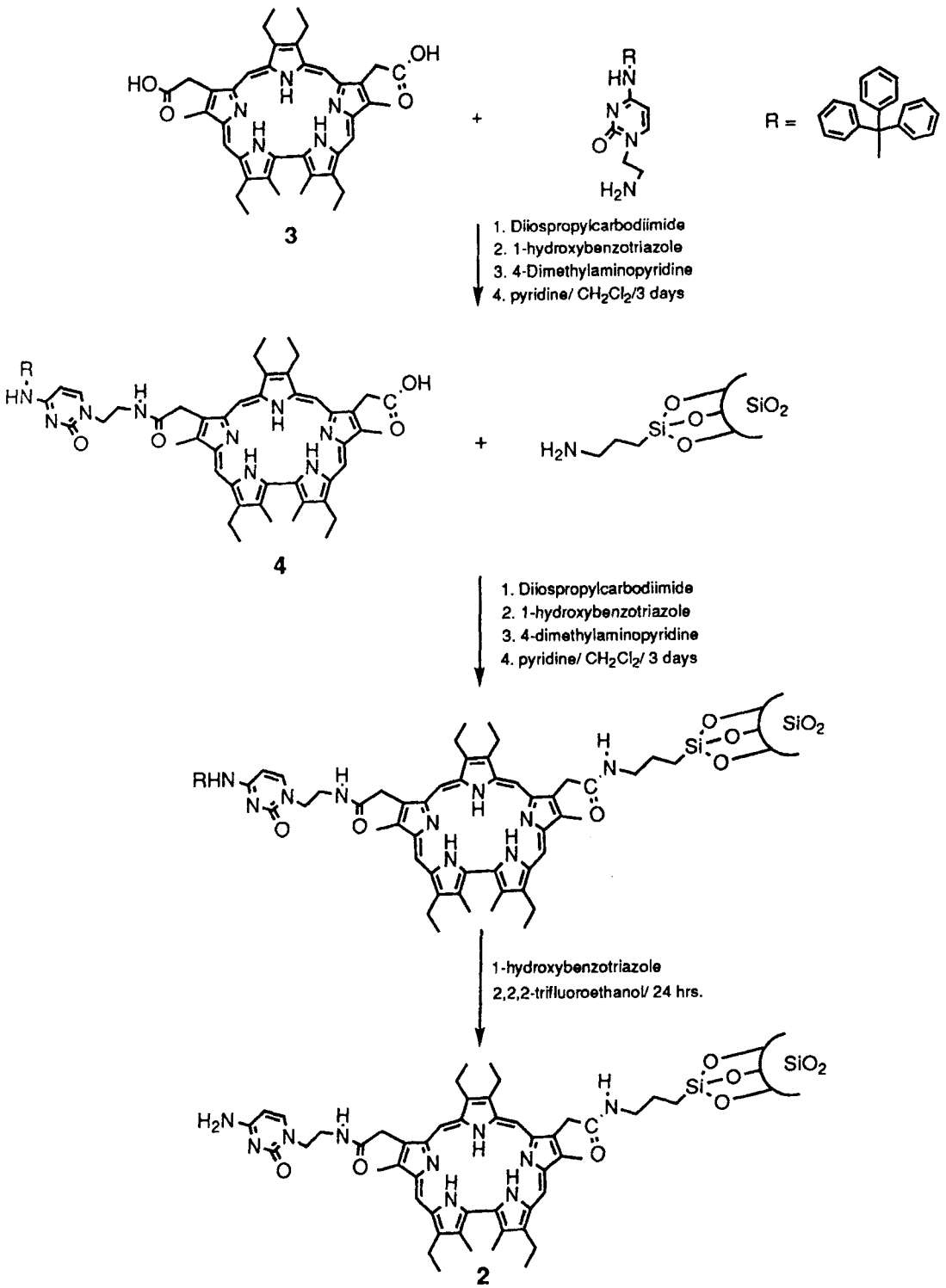


FIGURE 1 Synthesis of cytosine-sapphyrin substituted silica gel **2**.

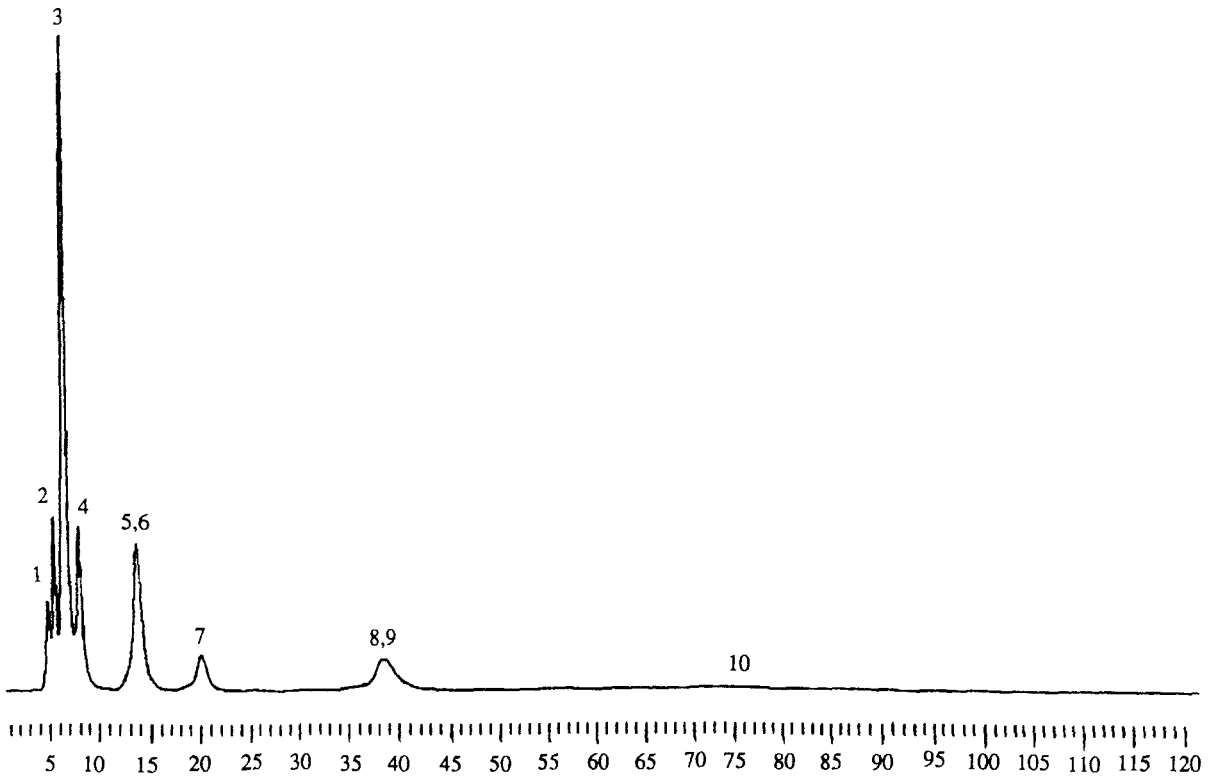


FIGURE 2 Separation of CMP (1), AMP (2), UMP (3), GMP (4), UDP (5), ADP (6), GDP (7), UTP (8), ATP (9) and GTP (10) on a column based on the cytosine-sapphyrin silica gel **2**: mobile phase, 25 mM phosphate, pH = 7.1; flow rate, 0.3 ml/min.; column temperature 25 °C; detection, 260 nm (0.100 absorbance units full scale (AUFS)).

case of the monophosphates, that all four nucleotides (CMP, GMP, AMP, UMP) could readily be separated from each other under isocratic conditions (See Figure 2). In particular, cytidine 5′-monophosphate elutes first and is found to be well separated from guanosine 5′-monophosphate (which elutes last). This is illustrated in Figure 4, a figure that represents an expanded view of Figure 2.

The good resolution seen in the case of the various non-guanine mononucleotides (XMP; X = A, C, U, G) is, unfortunately, not seen in the case of the di-, and trinucleotides (XDP and XTP; X = A, C, U) (c.f., Figures 5 and 6). As was true for the previously reported sapphyrin columns based on sapphyrin alone (i.e. **1**),[10] the bands ascribed to the multianionic species were found to be subject to peak broadening as the number of phosphate groups increased. While origins of this broadening phenomenon are not yet fully understood, they could reflect the intrinsic variations in affinity to which a multiply anionic substrate (e.g. GTP) would be subject at every monocationic sapphyrin receptor site.[17]

On the basis of the above findings, we conclude that cooperative hydrogen bonding interactions, derived in this instance from the nucleoside cytosine, in concert with anion chelation attractions established by a protonated sapphyrin center, can be used to effect the separation of a given class of nucleotides (e.g., GMP, GDP, GTP) from other naturally occurring nucleotides. These findings thus support the notion that rationally designed ditopic nucleobase-sapphyrin conjugates can be used to effect the specific binding of a chosen nucleotide species. They, therefore, point the way to the development of highly specific sapphyrin-derived solid supports.

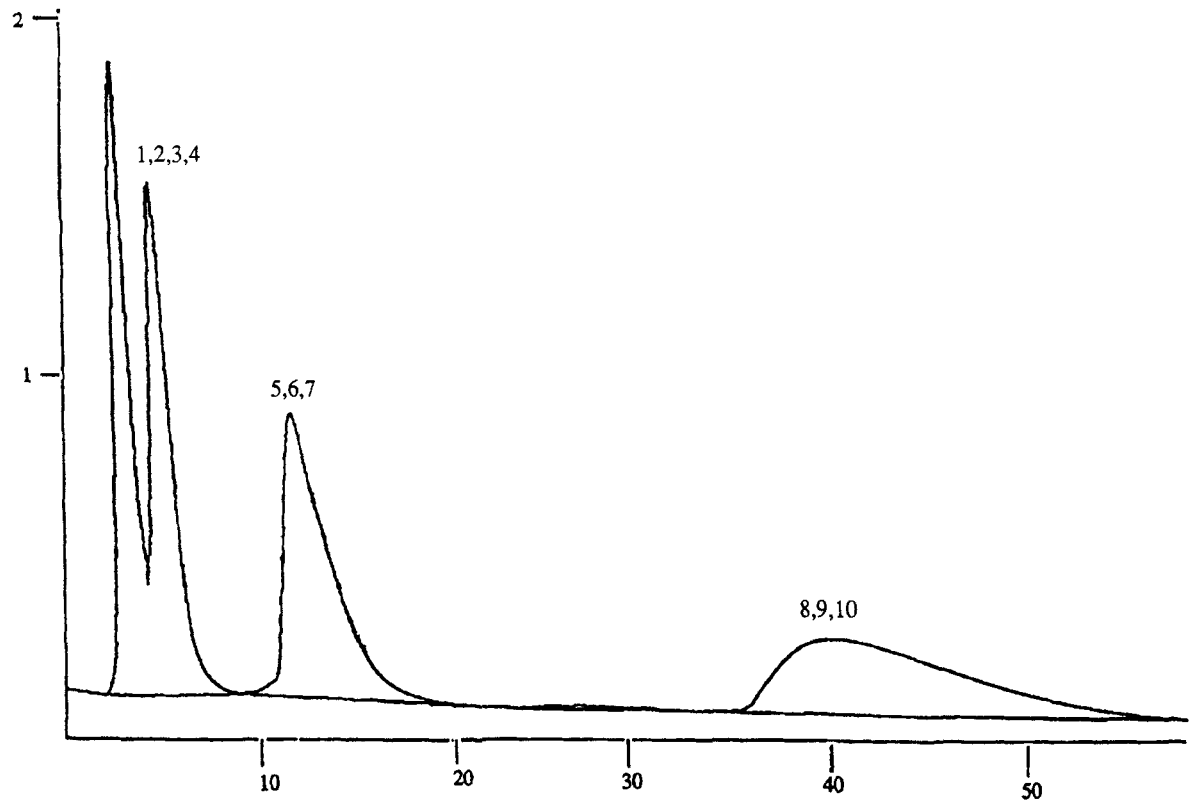


FIGURE 3 Separation of CMP (1), AMP (2), UMP (3), GMP (4), UDP (5), ADP (6), GDP (7), UTP (8), ATP (9) and GTP (10) on a column based on the sapphyrin silica gel **1**: mobile phase, 25 mM phosphate, pH = 7.1; flow rate, 0.2 ml/min.; column temperature 25 °C; detection, 260 nm (0.100 AUFS).

EXPERIMENTAL

Cytosine-Sapphyrin monoacid synthesis. The synthesis of the dicarboxylic acid derivative of sapphyrin **2** has been recently reported.[18]

Preparation of Cytosine-sapphyrin, 4 Sapphyrin-dicarboxylate **3** (59 mg, 0.089 mmols) and 1-hydroxybenzotriazole (4.5 mg, 0.033 mmol) were dissolved in dry DMF (50 ml). The solution was cooled to 0 °C and diisopropylcarbodiimide (112 mg, 0.89 mmols) was added. The solution was stirred for 40 minutes at which time, 4-dimethylaminopyridine (8 mg, 0.065 mmol), pyridine (0.5 ml, 6.18 mmol), and trityl protected N-ethylaminocytosine (24.5 mg, 0.0712 mmol) were added. The solution was stirred for 4 days at room temperature. Purification was made using column chromatography (silica gel; dichloromethane/ methanol/ trifluoroacetic acid, eluent) to yield 35 mg (37%) of product **3**. ^{1}H NMR (300 MHz, $CDCl_3$): δ −3.90 (s, 2H), −3.75 (s, 1H), 2.15 (12H, t), 3.25 (2H, t), 3.72 (4H, m), 3.83 (2H, t), 4.10 (6H, s), 4.20 (6H, s), 4.60 (8H, m), 5.00 (4H, m), 5.50 (1H, d), 6.83 (1H, d), 6.87 (1H, s), 7.5–7.6 (15H, m). ^{13}C NMR (300 MHz, $CDCl_3$): δ 12.60, 12.69, 12.90, 15.72, 15.89, 17.35, 17.55, 17.68, 20.69, 20.78, 21.01, 23.02, 37.01, 38.91, 48.51, 91.51, 91.92, 98.12, 98.31, 121.47, 126.35, 127.10, 127.85, 129.08, 129.91, 130.21, 130.26, 132.90, 132.99, 135.60, 137.41, 138.60, 140.50, 142.03, 142.10, 142.49, 147.09, 158.60, 173.65, 173.71. HRMS (FAB) for $C_{67}H_{71}N_9O_4$ (M^+) calcd 1065.595, found 1065.592.

Preparation of cytosine-sapphyrin-functionalized 3-aminoproyl silica gel, 2 3-Aminopropyl

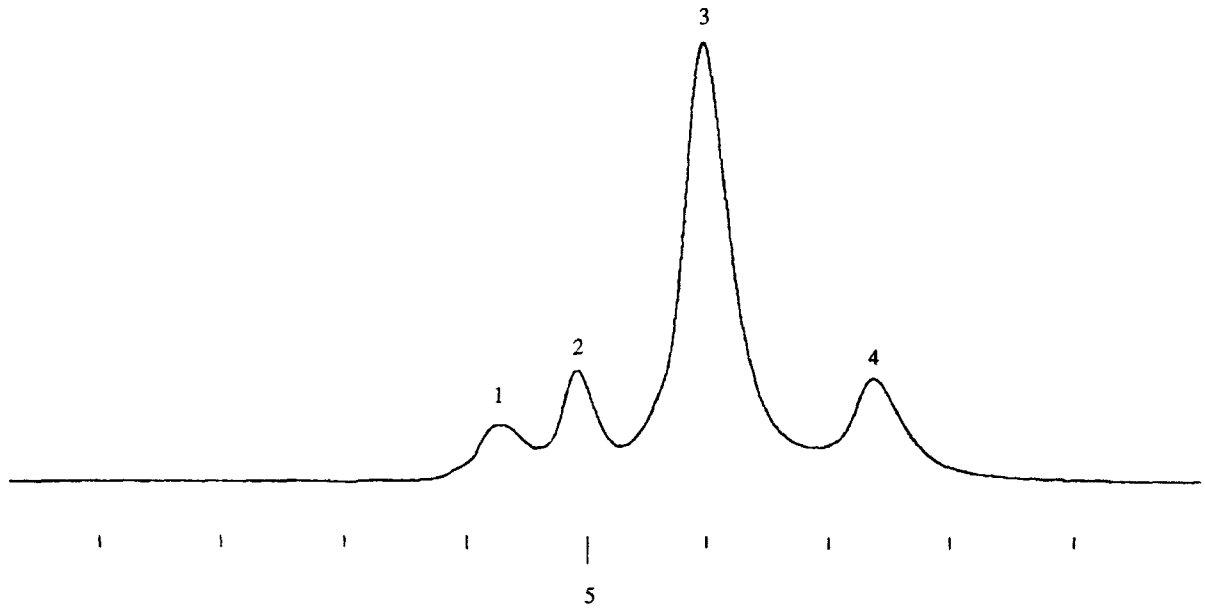


FIGURE 4 Expanded view of nucleotide monophosphate separation effected using a column based on the cytosine-sapphyrin silica gel **2**. CMP (1), AMP (2), UMP (3), GMP (4); mobile phase, 25 mM phosphate, pH = 7.1; flow rate, 0.3 ml/min.; column temperature 25 °C; detection, 260 nm (0.100 absorbance units full scale (AUFS)).

trimethyl silyl protected silica gel (1g) (Phase Separations, 1.0 mmol N per gram) was suspended in a solution of dry dichloromethane (25 ml), dry pyridine (0.5 ml, 6.18 mmol) and 4-dimethylaminopyridine (10 mg, 0.078 mmol). Cytosine substituted sapphyrin, **4**, was likewise dissolved in dry dichloromethane (25 ml) and activated with N,N'-diisopropylcarbodiimide (0.126 g, 1 mmol) and 1-hydroxybenzotriazole (5 mg, 0.037 mmol) at 0 °C for 40 min. This latter solution was then slowly added to the 3-aminopropyl silica gel slurry referred to above.

The resulting reaction mixture was then stirred at room temperature for five days. The silica gel was then filtered off, washed with dichloromethane (30 ml), methanol (50 ml), water (100 ml), methanol (50 ml) and dichloromethane (50 ml). It was then dried on high vacuum before being subjected to further derivatization. Towards this end, the triphenyl methyl group was removed from the cytosine portion of the product by suspending the sapphyrin substituted silica gel in 2,2,2-trifluoroethanol (30 ml) and 1-hydroxybenzotriazole (100 mg, 0.74 mmol) and stirring the solution for 2 days at room temperature.[19] The resulting product was the desired substituted silica gel **2**. It was collected by filtration and washed with methanol (50 ml) and dichloromethane (50 ml). It was then dried under high vacuum for two days prior to use. The isolated yield was 1.06 g. The microanalysis for **2**, (C 3.26%, H 0.87%, N 0.47%) proved similar to that of trimethylsilyl protected amino propyl silica gel, (C 3.13%, H 0.84%, N 0.47%), suggesting, as desired, a rather low sapphyrin loading level. Attachment of the cytosine substituted sapphyrin to the 3-amionopropyl silica gel was further confirmed by means of UV/Vis of spectroscopic analysis. This was done by comparing a slurry formed from silica gel **2** in chloroform to one containing the non-derivatized silica gel as a blank. Two broad peaks at 283 nm and 460 nm ascribed to the cytosine and monomerized sapphyrin subunit respectively, were observed.

Chromatography. All peak assignments were made by individual component injection as well as controlled co-injection.

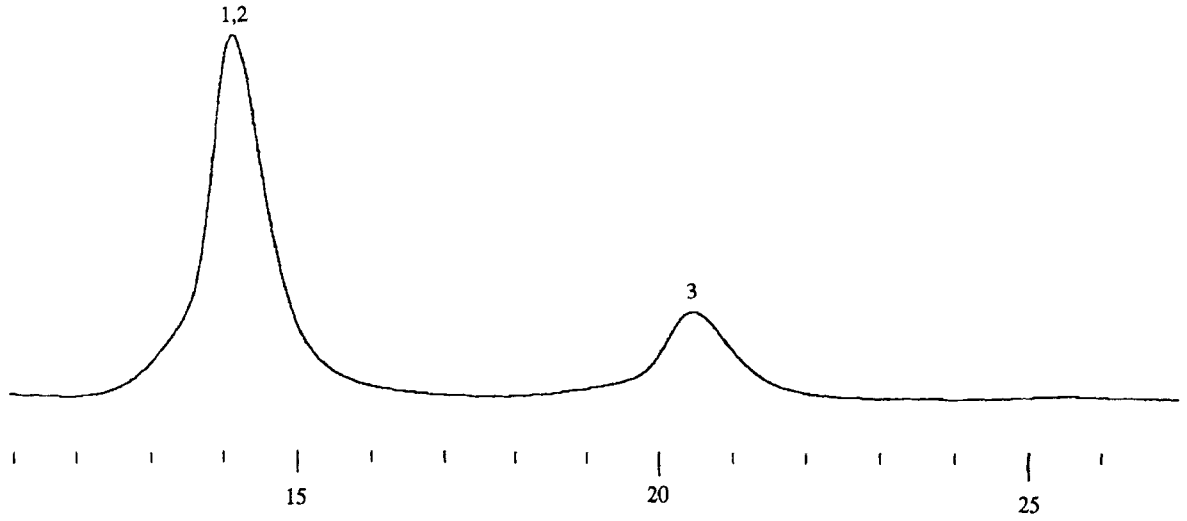


FIGURE 5 Expanded view of nucleotide diphosphate separation effected using a column based on the cytosine-sapphyrin silica gel **2**. UDP (1), ADP (2), GDP (3); mobile phase, 25 mM phosphate, pH = 7.1; flow rate, 0.3 ml/min.; column temperature 25 °C; detection, 260 nm (0.100 absorbance units full scale (AUFS)).

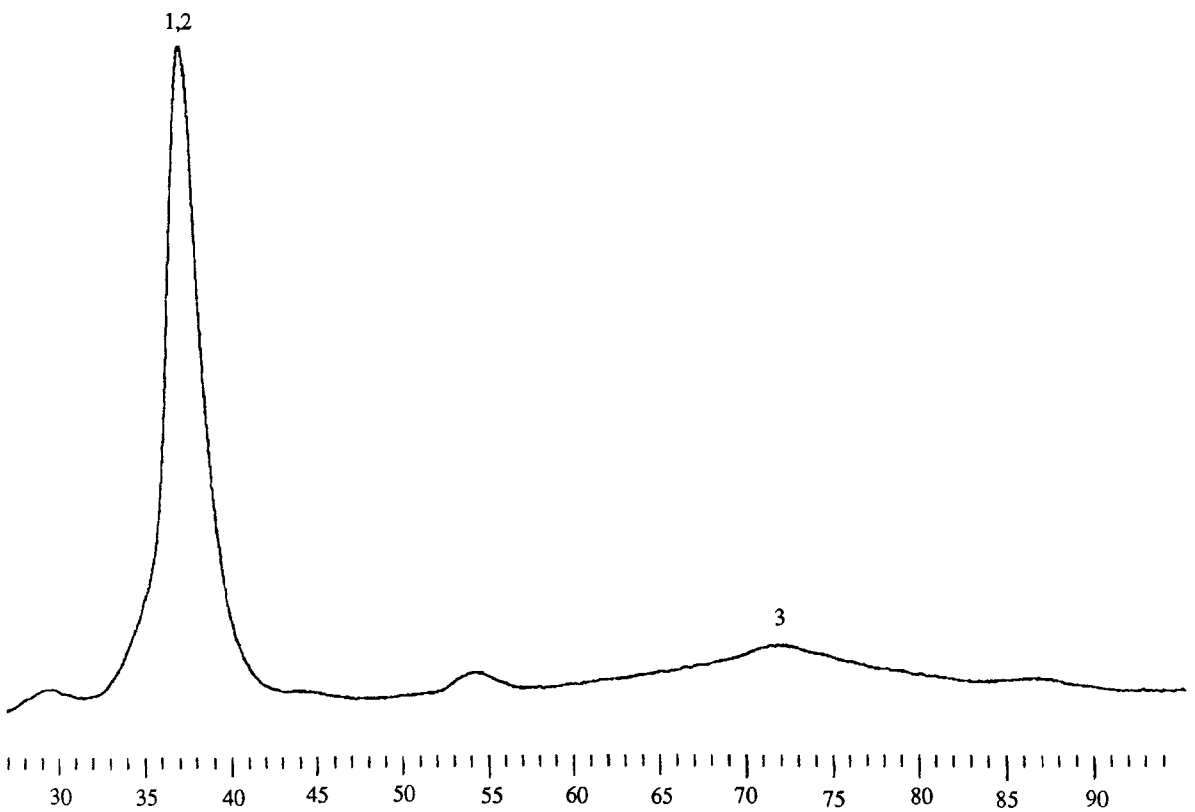


FIGURE 6 Expanded view of nucleotide triphosphate separation effected using a column based on the cytosine-sapphyrin silica gel **2**. UTP (1), ATP (2), GTP (3); mobile phase, 25 mM phosphate, pH = 7.1; flow rate, 0.3 ml/min.; column temperature 25 °C; detection, 260 nm (0.100 absorbance units full scale (AUFS)).

Instrumentation. HPLC analyses were made using a Varian (Walnut Creek, Ca.) 9002 solvent delivery system, a Varian 9050 variable wavelength UV-Visible detector, a Varian Star Chromatography Workstation-Version 3, and a Rheodyne (Cotati, CA) Model 7125 sample valve with a 20-μl loop.

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[16] All columns commericially packed by Alltech Associates Inc. (Deerfield, Il) The procedure is as follows; The cytosine-sapphyrin substituted silica gel (1–1.5 g) was slurried in 50% acetonitril/50% water (v/v). The slurry was then sonicated and transferred to a chromatography column packing system and packed into a 3.1 mm i.d. × 100 mm stainless steel HPLC column. After eluting the slurry-generating solvent, the column was removed from the packing device and the end fittings attached.

[17] This broadening is also apparent in the broadened nature of the GTP peak (see Figures 1 and 5) in comparison to that of GMP and GDP.

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