# Calix[4]pyrroles: New Solid-Phase HPLC Supports for the Separation of Anions**

## Jonathan L. Sessler,* Philip A. Gale, and John W. Genge

**Abstract:** The preparation of two distinct calix[4]pyrrole-modified silica gels is reported. These systems, designed to investigate the binding characteristics of calix[4]pyrroles with anionic and neutral substrates, also provide a new solid support for the HPLC separation of nucleotides, oligonucleotides, N-protected amino acids and perfluorinated biphenyls. Binding affinities for the interaction of anions with calix[4]pyrrole amide derivatives are also reported; these were determined from $^1$H NMR spectroscopic analyses carried out in $CD_2Cl_2$.

## Introduction

The covalent attachment of molecular receptors to solid supports provides a time-honored means of exploring receptor–substrate interactions.[1–5] Pioneering work in this area by Cram and co-workers produced resins, covalently modified with chiral crown ethers, that proved capable of resolving enantioselectively various amino acid derivatives.[1] Other systems (most notably those of Izatt, Bradshaw, and Christensen),[2] consisting of azacrown ethers attached to silica gels, have proven useful in the separation of mixtures of metal cations. In this classic work and in other instances[3] this approach has allowed binding interactions involving a large number of substrates to be analyzed under identical experimental conditions. As such, it provides a convenient and well-validated method of quickly testing the substrate-binding potential of a new receptor or proposed receptor.

The above approach has been extensively exploited in the area of cation recognition and separation; however, it has been less frequently applied in the study of anion binding. One such approach that appears attractive involves the attachment of a metallated porphyrin to either polystyrene or silica gel;[6, 7] these systems have been employed in the separation of quite a number of anionic species including iodide, thiocyanate, and benzoic acid derivatives. We have

also introduced another anion-separating system that consists, in general terms, of various sapphyrin-modified silica gels (for example gel S). In this case, the resulting solid supports allow the HPLC separation of anions such as phenyl arsenate,



**Gel S**
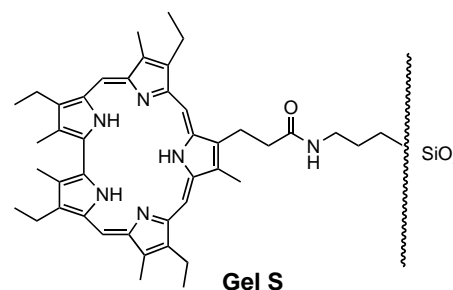
benzenesulfonate, and benzoate, as well as mixtures of nucleotides.[8, 9] However, the observation of broad peaks in the case of short (3- to 9-mer) oligonucleotide mixtures as well as the synthetic difficulties associated with preparing functionalized sapphyrins prompted us to search for an alternative chelating group upon which the construction of the anion-separating silica gels could be based. As detailed in this report, the calix[4]pyrroles show promise in this regard.

The calix[4]pyrroles (*meso*-octaalkylporphyrinogens) are a class of nonaromatic macrocycles first synthesized by Baeyer in 1886[10] that act as effective and selective anion-binding agents both in solution and in the solid state.[11–13] In this paper we show two ways of attaching these easily made macrocycles to aminopropyl silica gel. We also show that the resulting supports (gel M and gel B) make HPLC columns that have quite intriguing anion-separating properties.

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**Gel M**          **Gel B**
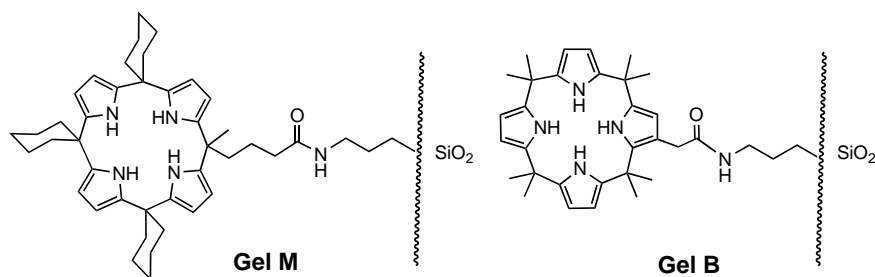
## Results and Discussion

The key precursors to gel M and gel B, the functionalized *meso*-hook and *β*-hook calix[4]pyrrole esters **1** and **2**, respectively, were synthesized from readily available starting materials (i.e. cyclohexanone, pyrrole, and methyl-4-acetyl-butyrate in one step for **1** and acetone, pyrrole, *n*-butyllithium, and ethyl bromoacetate in two steps for **2**), as previously described.[12, 13] These molecules were de-esterified to produce the calix[4]pyrrole monoacids **3** and **4**, respectively. These



**1**          **2**



**3**          **4**
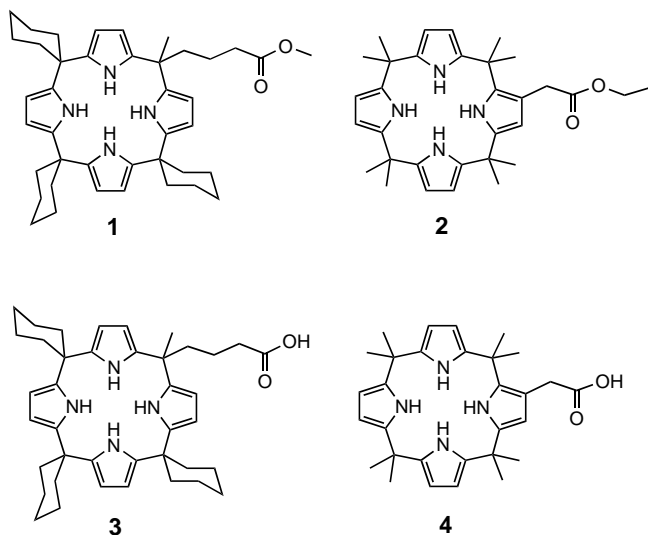
latter materials were then coupled to aminopropyl silica gel with standard amide-coupling conditions[14] to produce two new silica gel solid supports containing amidocalix[4]pyrrole groups [gel M (*meso*-hook) and gel B (*β*-hook)]. Model compounds **5** and **6** were synthesized by coupling acids **3** and **4** with *n*-butylamine in the presence of (1-benzotriazol-yl)oxy tris(dimethylamino)phosphonium hexafluorophos-phate (BOP PF$_6$).[15] These latter materials were used to study



**5**          **6**
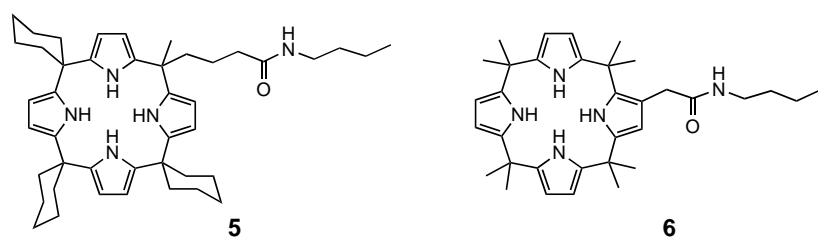
the anion-binding properties of appropriately functionalized amidocalix[4]-pyrrole controls in solution.

Initial experiments (using either UV or conductance detection of anions) proved successful, with both gels B and M selectively retaining a) fluoride, chloride, bromide, hydrogensulfate, and dihydrogenphosphate (see Table 1), b) phenyl arsenate, phenyl phosphate, and phenyl sulfonate (Table 1), c) Cbz-protected anionic amino acids (serine, glutamine, alanine, phenylalanine, tryptophan, aspartate, and glutamate, Figure 1).

Table 1. Retention of anions by gels B and M.

| Anion | Elution times [min] | |
| --- | --- | --- |
| | Silica gel B | Silica gel M |
| chloride[b] | 17.9 ($\pm$0.1)[a] | 15.2 ($\pm$0.1)[a] |
| dihydrogenphosphate[b] | 22.0 ($\pm$0.1)[a] | 20.1 ($\pm$0.1)[a] |
| hydrogensulfate[b] | 16.2 ($\pm$0.1)[a] | 16.2 ($\pm$0.2)[a] |
| fluoride[b] | 16.9 ($\pm$0.1)[a] | 16.4 ($\pm$0.2)[a] |
| phenyl arsenate[c] | n.d.[d] | 4.9 ($\pm$0.1) |
| benzoate[c] | n.d.[d] | 6.9 ($\pm$0.1) |
| benzenesulfonate[c] | n.d.[d] | 7.0 ($\pm$0.1) |
| phenyl phosphate[c] | n.d.[d] | 15.1 ($\pm$0.1) |

[a] Times given for individual elution of tetrabutylammonium anions. [b] Anions were eluted as 1 mM CH$_3$CN solutions of their tetrabutyl-ammonium salts under the following conditions: mobile phase CH$_3$CN; flow rate 0.40 mL min$^{-1}$; detection by conductivity; column temperature 25 °C. [c] Anions were eluted as 1 mM aqueous solutions under the following conditions: mobile phase 50 mM phosphate buffer, pH = 7.0, flow rate 0.3 mL min$^{-1}$, column temperature 25 °C, detection UV = 254 nm. [d] n.d. = not determined.
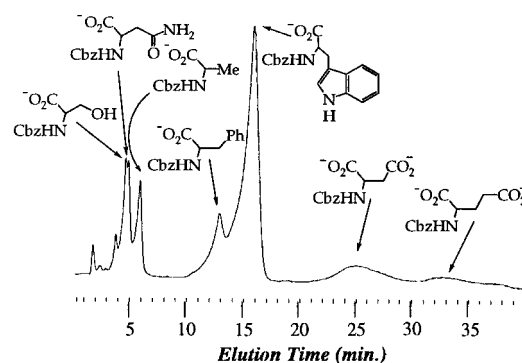


Figure 1. HPLC separation of Cbz-N-protected amino acids on a calix-pyrrole-modified silica gel column, prepared from gel M. Flow rate 0.3 mL min$^{-1}$, mobile phase 70/30, 30 mM acetate buffer at pH 7.0/acetoni-trile (*v/v*) (isocratic), column temperature 25 °C, UV detection at 254 nm.

The next set of substrates tested were the phosphorylated derivatives of adenine. The results are illustrated by Figure 2, which shows the HPLC separation of 5′-adenosine monophosphate (AMP), 5′-adenosine di-phosphate (ADP) and 5′-adenosine triphos-phate (ATP) on gel M. The order of elution differs from that obtained on other neutral solid supports used for anion separations,

including standard reverse-phase liquid chromatography,[16] in that the more highly charged nucleotide is retained longer without the use of ion-pairing agents. This is presumably due to the higher charge density present in the di- and triphosphate anions, which permits these substrates to interact more favorably with the calix[4]pyrrole subunits present on the column. Thus, the greater the number of phosphate groups, the longer the retention time.
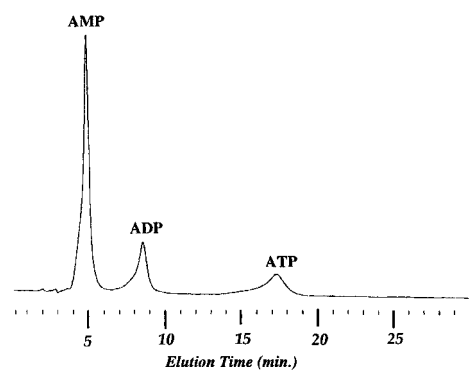


Figure 2. HPLC separation of AMP, ADP, and ATP on the calixpyrrole column derived from gel M. Flow rate 0.3 mL min⁻¹, mobile phase 200 mm sodium phosphate (isochratic), pH 7.0, column temperature 25 °C, UV detection at 262 nm.

On the basis that the above suppositions are true, it is predicted that silica gels M and B should function as efficient supports for the separation of medium-length oligonucleotides, molecules that are important in both molecular biology and medicine (e.g. antisense technologies).[17] Figure 3 shows the separation on gel B of a mixture of unprotected oligodeoxyadenylate fragments (dA) containing between 12 and 18 nucleotide subunits. As expected, the species containing
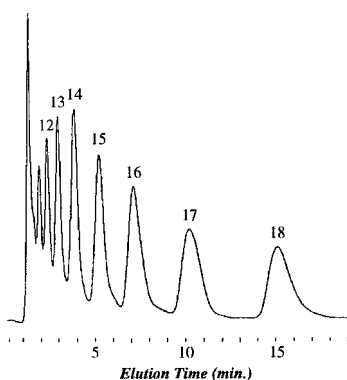


Figure 3. HPLC separation of dA₁₂₋₁₈ on the calixpyrrole column derived from gel B. Flow rate 0.4 mL min⁻¹, mobile phase 50/50, CH₃CN/50 mm sodium chloride:40 mm sodium phosphate (v/v) (isochratic), pH 7.0, column temperature 25 °C, UV detection at 265 nm.

the greater numbers of phosphate groups display the longer retention times, a result that was confirmed in studies involving a similar mixture of unprotected oligodeoxythymidylates (data not shown). Interestingly, both gels B and M were found to be capable of separating three oligonucleotide hexamers of equal charge and length (Figure 4). In these cases
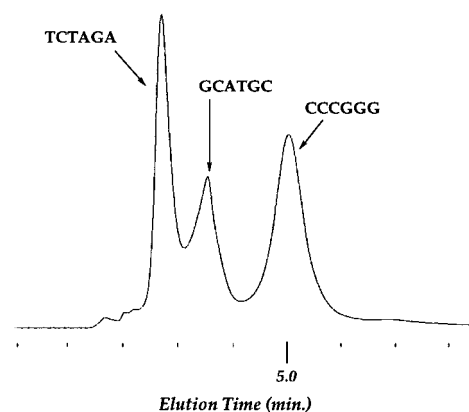


Figure 4. HPLC separation of hexamers: TCTAGA, GCATGC, and CCCGGG on the calixpyrrole column derived from gel M. Flow rate 0.4 mL min⁻¹, mobile phase 50/50, CH₃CN/50 mm sodium phosphate buffer (v/v) (isochratic), pH = 7.0, column temperature 25 °C, UV detection at 265 nm.

the separation presumably reflects the different number of hydrogen bonds that are possible for the various nucleobases of which these oligonucleotides are comprised. This critical finding supports the contention that calixpyrrole-based solid supports could provide a new nonelectrophoretic HPLC method for anionic and oligonucleotide separations that could usefully complement reverse-phase,[18] ion-pair,[19] size-exclusion,[20] or ion-exchange chromatography,[21] as well as capillary electrophoresis.[22] The ease of synthesizing a functionalized calixpyrrole and the fact that unprotected oligonucleotides may be separated are possible advantages, especially with nonanalytical (i.e. preparative) applications.

It quickly became apparent that calix[4]pyrrole silica gels are more easily synthesized than the corresponding sapphyrin-based systems; however, we were also interested in ascertaining whether gels M and B in fact performed better when used as anion-separating HPLC supports. We therefore chose to investigate the separation of the same unprotected mixture of oligodeoxyadenylate 12–18 mers using the sapphyrin gel S (Figure 5) as previously studied with the calix[4]pyrrole support gel B (Figure 3). Comparison of Figures 3 and 5 shows that, under identical conditions of elution, the sapphyrin column fails to effect a clean separation, whereas gel B clearly does. While not a complete proof of



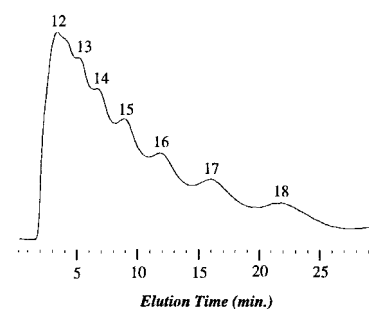Figure 5. HPLC separation of dA₁₂₋₁₈ on the sapphyrin column derived from gel S. Flow rate 0.4 mL min⁻¹, mobile phase 50/50, CH₃CN/50 mm sodium chloride: 40 mm sodium phosphate (v/v) (isochratic), pH 7.0, column temperature 25 °C, UV detection at 265 nm.

superiority, the apparently improved separation observed with the calixpyrrole support can be rationalized in terms of the different binding characteristics involved. Specifically, 3,12,13,22-tetraethyl-2,7,18,23-tetramethyl-8,17-di(hydroxy-propyl)sapphyrin[23] shows a higher affinity for the phosphate anion ($K_a = 1 \times 10^4$ M$^{-1}$ in methanol) than $\beta$-hydroxycarbonyl-methyl-*meso*-octamethylcalix[4]pyrrole **4** ($K_a < 15$ M$^{-1}$ in methanol).[24] The higher affinity of sapphyrin towards anionic species presumably hinders the release of the anion from the sapphyrin subunit, thereby causing broad, ill-defined peaks.

To further assess the extent to which binding affinities correlate with observed separation efficiencies, the chloride-, dihydrogenphosphate-, and hydrogensulfate-binding properties of compounds **5** and **6** were analyzed in solution. The relevant studies were carried out in CD$_2$Cl$_2$ (for reasons of solubility) with the stability constants and binding stoichiometries (1:1 in all instances) being determined by $^1$H NMR titration.[25] As can be deduced from the results in Table 2, these analyses do in fact provide a basis for understanding the calix[4]pyrrole-related HPLC results discussed above. For instance, on both silica gels M and B, phenyl phosphate is retained on the column longer than, and hence readily separated from, benzenesulfonate; this can be explained in terms of the fact that dihydrogenphosphate forms a stronger bond to the model compounds **5** and **6** than hydrogensulfate.[26] Likewise, similar retention times are observed when either gel M or B is used to separate, for example, H$_2$PO$_4^-$; this could reflect the fact that nearly identical $K_a$'s for this substrate are displayed by both **5** and **6** in CD$_2$Cl$_2$ solution.

Table 2. The anion-binding properties of compounds **5** and **6** in CD$_2$Cl$_2$ solution.

| Anion[a] | Stability constants [M$^{-1}$] | |
|---|---|---|
| | Compound **5** | Compound **6** |
| Chloride | 415 ($\pm$45) | 405 ($\pm$10) |
| Dihydrogenphosphate | 62 ($\pm$6) | 80 ($\pm$15)[b] |
| Hydrogensulfate | <10 | <10 |

[a] Anions were added as 0.1 M CD$_2$Cl$_2$ solutions of their tetrabutylammonium salts to 10 mM solutions of the receptor in CD$_2$Cl$_2$ with concentration changes being accounted for by EQNMR.[25] In determining the stability constants, the possible effects of ion pairing (if any) were ignored. [b] Estimated value. The NH proton resonance broadened considerably during the titration, forcing manual notation of the resonance frequency. This value should, therefore, be treated with caution.

In addition to binding anions, calix[4]pyrroles are also known to bind neutral substrates, albeit weakly.[29] Since weak binding interactions can lead to good HPLC separations,[30] we explored whether supports such as M and B could also be used in neutral substrate separation. As a preliminary test we chose to investigate the separation of polyfluorobiphenyls; these compounds serve as models for the analogous, albeit much more toxic, polychlorobiphenyls (PCBs). As illustrated in Figure 6, these fluorinated species are readily purified on column B with the more highly substituted substrate perfluorobiphenyl eluted last (similar results were obtained with column M). Such findings are consistent with a mechanism in which separation is achieved, at least in part, on the basis of hydrogen-bonding interactions. In particular, the increased
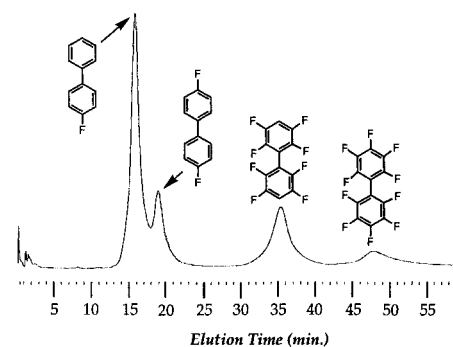


Figure 6. HPLC separation of polyfluorobiphenyls on the calixpyrrole column derived from gel B. Flow rate 0.2 mL min$^{-1}$, mobile phase 76/24 H$_2$O/CH$_3$CN ($v/v$), column temperature 25 °C, UV detection at 254 nm.

polarization arising from the presence of the electronegative fluorine substituents allows stronger calixpyrrole – substrate interactions which are analogous to those observed with polyanionic oligonucleotide species. We therefore believe that columns M and B could separate a wide range of both neutral and anionic substrates, provided that the substrates differ considerably in their overall polarity.

## Experimental Section

Compounds **1**, **2**, and **3** were synthesized by previously published procedures.[12, 13]

***β*-Hydroxycarbonylmethyl-*meso*-octamethylcalix[4]pyrrole** (**4**): Compound **2** (200 mg, 0.38 mmol) was stirred in 20 mL of ethanol. This slurry was heated to reflux and NaOH$_{aq}$ (20 mL, 2.0 M) was added. The reaction mixture was heated under reflux until all the ester had been consumed (approximately 4 hours). The ethanol was then removed in vacuo and a further 50 mL of cold water added to the solution. The solution was then acidified with concentrated perchloric acid to pH 1. The acid **4** precipitated out as a white powder and was collected by filtration and dried under high vacuum, yielding 146 mg (79 %). $^1$H NMR (500 MHz, CD$_2$Cl$_2$): $\delta = 7.84$ (s, 1H, NH), 7.24 (s, 1H, NH), 7.01 (coincident s, 2H, NH), 5.90 – 5.76 (overlapping m, 5H, CH$_{py}$), 3.71 (s, 2H, OCH$_2$), 1.51 (s, 6H, CCH$_3$), 1.50 (s, 6H, CCH$_3$), 1.48 (s, 6H, CCH$_3$), 1.47 (s, 6H, CCH$_3$); $^{13}$C NMR (500 MHz, CD$_2$Cl$_2$): $\delta = 179.13$ (C=O), 139.96 (C$_{py}$), 139.20 (C$_{py}$), 139.09 (C$_{py}$), 138.60 (C$_{py}$), 138.52 (C$_{py}$), 137.45 (C$_{py}$), 133.96 (C$_{py}$), 109.27 (C$_{py}$), 107.11 (CH), 103.21 (CH), 103.14 (CH), 103.05 (CH), 102.91 (CH), 102.75 (CH), 102.14 (CH), 37.20 (CCH$_3$), 35.49 (CCH$_3$), 35.40 (OCH$_2$), 35.23 (CCH$_3$), 33.68 (CCH$_3$), 30.38 (CCH$_3$), 29.16 (CCH$_3$), 29.09 (CCH$_3$), 29.00 (CCH$_3$), 28.85 (CCH$_3$); HRMS (FAB) calcd for C$_{30}$H$_{38}$N$_4$O$_2$: 486.2989, found: 486.2995 ($\Delta$ 1.1 ppm).

***meso*-Butylamidecalix[4]pyrrole** (**5**): Compound **3** (*meso*-hook acid, 268 mg, 0.43 mmol) was dissolved in dry DMF (20 mL) under an argon atmosphere. *n*-Butylamine (31.6 mg, 0.43 mmol) was added followed by BOP PF$_6$ (230 mg, 0.52 mmol) and triethylamine (87 mg, 0.86 mmol). The reaction mixture was stirred for 48 hours. The DMF was then removed in vacuo and the product was purified by silica column chromatography (SiO$_2$, CH$_2$Cl$_2$:CH$_3$OH 99:1 eluent), which yielded a white foam (118 mg, 41 %). $^1$H NMR (250 MHz, CD$_2$Cl$_2$): $\delta = 7.26$ (s, 2H, NH), 7.17 (s, 2H, NH), 5.93 (s, 2H, CH$_{py}$), 5.92 (s, 2H, CH$_{py}$), 5.90 (s, 2H, CH$_{py}$), 5.89 (s, 2H, CH$_{py}$), 5.46 (br t, 1H, NH$_{amide}$), 3.17 (q, J=6.3 Hz, 2H, CH$_2$), 1.87 (br coincident resonances, 18H, 9 CH$_{2cyclohexyl}$), 1.45 (br coincident resonances, 25H, 6 CH$_{2cyclohexyl}$ + 5 CH$_{2hook}$ + CH$_{3hook}$), 0.92 (t, J=7.2 Hz, 3H, CH$_3$); $^{13}$C NMR (62.90 MHz, CD$_2$Cl$_2$): $\delta = 172.6$, 137.6, 137.2, 137.0, 136.9, 104.0, 103.9, 103.6, 40.0, 39.8, 39.5, 39.4, 38.8, 37.3, 37.0, 36.7, 32.2, 26.4, 25.6, 23.2, 20.9, 20.5, 14.0; HRMS (FAB) calcd for C$_{44}$H$_{61}$N$_5$O: 675.4876, found: 675.4879 ($\Delta$ 1.2 ppm).

***β*-Butylamidecalix[4]pyrrole** (**6**): Compound **4** ($\beta$-hook acid, 300 mg, 0.62 mmol) was dissolved in dry DMF (20 mL) under an argon atmosphere.

n-Butylamine (45 mg, 0.62 mmol) was added, followed by BOP$\cdot$PF$_6$ (327 mg, 0.74 mmol) and triethylamine (125 mg, 1.2 mmol). The reaction mixture was stirred for 48 hours. The DMF was then removed in vacuo and the product was purified by silica column chromatography (SiO$_2$, CH$_2$Cl$_2$:CH$_3$OH 99:1 eluent), which yielded a white foam (331 mg, 99 %). $^1$H NMR (250 MHz, CD$_2$Cl$_2$): $\delta$ = 8.80 (s, 1 H, NH), 7.24 (s, 1 H, NH), 7.01 (coincident s, 2 H, NH), 5.92–5.82 (m, 7 H, 6CH$_{py}$ and NH$_{amide}$), 5.70–5.69 (1 H, d, CH$_{py}$), 3.51 (s, 2 H, OCH$_2$), 3.29 (q, $J$ = 6.2 Hz, 2 H, CH$_2$), 1.66–1.30 (coincident resonances, 28 H, 8 CH$_{3\,meso}$, 2 CH$_{2\,hook}$), 0.96 (t, 3 H, CH$_{3\,hook}$); $^{13}$C NMR (62.9 MHz, CD$_2$Cl$_2$): $\delta$ = 173.0, 140.2, 139.7, 139.2, 138.9, 138.4, 138.1, 137.4, 134.2, 110.7, 106.9, 103.2, 103.2, 103.1, 102.7, 102.5, 101.6, 39.7, 37.1, 35.9, 35.5, 35.4, 35.2, 32.1, 29.2, 29.0, 28.7, 20.5, 14.0; HRMS (FAB) calcd for C$_{34}$H$_{47}$N$_5$O: 541.3781, found: 541.3782 ($\Delta$ 0.2 ppm).

**Silica gel M**: Compound **3** (89 mg, 0.14 mmol) was dissolved in dry dichloromethane (100 mL) under an argon atmosphere and cooled to 0 °C. 1-Hydroxybenzotriazole (HOBT) (4 mg, 0.029 mmol) and diisopropylcarbodiimide (300 µl, 1.91 mmol) were added and stirred for 40 minutes at 0 °C. Trimethylsilyl-protected aminopropyl silica gel (3 g, 2% $w/w$), 4-dimethylaminopyridine (10 mg, 0.081 mmol), and dry pyridine (2 mL, 5.5 mmol) were then added and stirred at room temperature for 4 days. The product was filtered and washed with dichloromethane (200 mL), methanol (400 mL), water (100 mL), and acetonitrile (250 mL). The silica gel was dried in vacuo for two days and was then resuspended in dry dichloromethane (100 mL) under an argon atmosphere. The suspension was cooled to 0 °C. Dry pyridine (5 mL, 13.9 mmol) and acetyl chloride (1 mL, 11.4 mmol) were added and the slurry was stirred at room temperature for 24 hours. The silica gel was filtered and washed using the same procedure described above, and then dried for 24 hours in vacuo and packed into 3.2 × 100 mm HPLC columns by Alltech Associates (Deerfield, IL). Efficiency (plates/m$^2$): 14613. Elemental analysis: trimethylsilyl-protected silica gel: C 3.13 %, H 0.84 %, N 0.49 %; calixpyrrole-substituted silica gel: C 4.23 %, H 0.87 %, N 0.74 %.

**Silica gel B**: The procedure for the preparation of the modified silica gel was similar to the procedure described above, but using compound **4** (100 mg, 0.20 mmol) instead of compound **3**. Efficiency (plates/m$^2$): 39058. Elemental analysis: trimethylsilyl-protected silica gel: C 3.13 %, H 0.84 %, N 0.49 %; calixpyrrole-substituted silica gel: C 3.96 %, H 0.85 %, N 0.76 %.

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