Functionalized Calixspherands: Synthesis and Peptide Coupling

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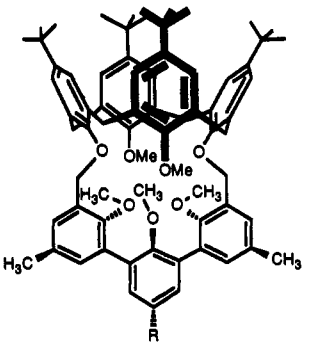
Calixspherands, like **1**, form kinetically stable complexes with alkali metal cations. For practical *in vivo* applications coupling of these complexes with carrier molecules is mandatory. Therefore, a general method for the synthesis of functionalized calixspherand **17** was developed starting from functionalized *m*-terphenyl **10** and *p-tert*-butylcalix[4]arene (**14**). The functionalized *m*-terphenyl was synthesized by a Suzuki–cross-coupling reaction. Functionalized calixspherand **17** has been coupled to a low molecular weight protein.

Introduction

Radioactive isotopes are often used in nuclear medicine for diagnosis and therapy.[1] We are especially interested in the use of rubidium-81 for the determination of blood flow in tissues and organs, in particular the kidneys, by the rubidium/krypton ratio method.[2] Practical application of this method for the monitoring of changes in the renal blood flow, however, is hampered by the redistribution of ^{81}RbCl over other tissues.[2] The method may be improved by the complexation of rubidium in a macrocyclic ligand and subsequent transport of the complex to the kidneys. Recently we have demonstrated that calixspherands form kinetically stable complexes with alkali metal cations.[3] The kinetic stability of the rubidium complexes appears sufficiently high for the practical application of the complexes *in vivo*. However, the calixspherand complexes themselves are not organ specific, and for practical use coupling to an organ-specific carrier is mandatory.

The calixspherands[4] synthesized so far (e.g. **1**, Chart 1) lack a functional group for the coupling to an organ-specific compound. Since the calixspherands are constructed from a calix[4]arene and a *m*-terphenyl moiety, the functional group can be introduced in either one of these building blocks. Despite all the work on the modification of calixarenes,[5] only a limited number of synthetic methods is available for the selective introduction of a functional group on the upper rim of a calix[4]arene.[6] In contrast, the functional groups can be simply introduced in the *m*-terphenyl moiety.[7,8] Cram and co-workers[8] introduced a nitro group in the *m*-terphenyl *via* a double Suzuki coupling.[9] This route allows the introduction of other functional groups such as ethers, esters, nitriles, amides, amines, and thioethers because these are compatible with the conditions of the Suzuki coupling.[10]

Chart 1



1 R = CH_3

A low-molecular-weight protein (LMWP) was selected as the organ-specific compound. Such a protein is kidney specific, thus allowing the calixspherand and the entrapped Rb^+ to be delivered to the kidneys. LMWPs accumulate rapidly, and to a high degree, in the proximal tubule cells of the kidney.[11a] In the lysosomes of these cells LMWPs are hydrolyzed into their single amino acid constituents.[11b] Egg-white lysozyme was chosen as LMWP because it has been successfully used in renal drug targeting.[12] Lysozyme contains 149 amino acids (MW 14400) and has seven free

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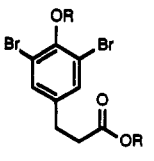
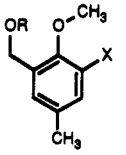
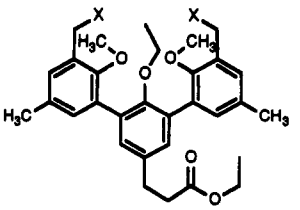
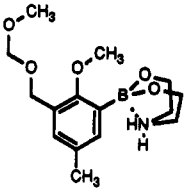
Chart 2









2 R = H
3 R = Et

4 R = Me X = Br
5 R = Me X = $B(OH)_2$
6 R = H X = Br
7 R = MOM X = Br
8 R = MOM X = $B(OH)_2$

9 X = OMe
10 X = Br
11 X = OMOM
12 X = OH

13

amino groups, present as aliphatic ϵ-amino groups of lysine and as the α-amino group of the *N*-terminal amino acids, to which the calixspherand can be coupled.[13]

In the present paper the synthesis of functionalized calixspherands[4] able to complex rubidium is described, as well as the coupling of such a ligand to lysozyme.

Results and Discussion

Synthesis. The carboxylic acid functional group was selected for coupling of the calxspherands[14] to LMWPs because good results were obtained for the coupling of drugs with a carboxylic acid group to LMWPs[12] *via* an active ester.[15,16] The carboxylic acid function is not inert under the entire reaction sequence for the synthesis of calixspherands. Therefore, it was necessary to start the synthesis with an appropriate precursor, viz. an ester group.

To increase the reactivity in the peptide coupling (*vide infra*) it was decided to use a 3-phenylpropionic acid instead of a benzoic acid derivative as central aromatic ring of the functionalized *m*-terphenyl. Moreover we have chosen for an ethoxy group instead of a methoxy group on the central ring of the *m*-terphenyl moiety in order to obtain a higher kinetic stability of the rubidium complexes for *in vivo* use.[3] The functionalized *m*-terphenyl was synthesized by a 2-fold Suzuki coupling between arylboronic acid **5** and 2,6-dibromo-1-ethoxybenzene (**3**). Thus, 3-(4-hydroxyphenyl)propionic acid was brominated in CH_2Cl_2 to give **2**[17] (Chart 2) in 88% yield. Subsequent ethylation with Et_2SO_4 afforded ethyl ester **3** in 89% yield. Boronic acid **5** (Chart 2) was prepared *via* a modified procedure of Cram and co-workers from bromoanisole **4** by bromo–lithium exchange and subsequent reaction with $(CH_3O)_3B$.[8] Bromoanisole **4** was prepared from 4-methylanisole via chloromethylation and bromination,[18] followed by reaction with sodium methoxide. In the next step **3** was coupled with boronic acid **5** to obtain *m*-terphenyl **9** in 85% yield. Attempts to substitute the benzylic methoxy groups for bromides, with HBr gas, resulted in the isolation of a mixture of compounds from which no pure **10** could be isolated. Thus the synthetic route was modified so that the bromides could be introduced by substitution of hydroxyl groups. There are some precedents in literature for this type of substitution.[19] In the modified procedure, the benzylic alcohol function in **6**[18] was protected with a methoxymethyl (MOM) group to afford **7**[20] in 88% yield (Chart 2). Bromoanisole **7** was than converted to boronic acid **8** (83%) by bromo–lithium exchange followed by reaction with $(CH_3O)_3B$ and acidic workup (pH ~6.5).[10a] A small quantity was converted to diethanolamine ester **13**[10a,21] for characterization. The remaining material was used as such in the Suzuki coupling with ester **3**. The crude *m*-terphenyl **11** was directly deprotected with a catalytic amount of concentrated aqueous HCl in methanol[22] to obtain *m*-terphenylbis(methanol) **12** in 50% overall yield. Subsequent treatment of **12** with PBr_3 afforded the desired bis(bromomethyl)terphenyl **10**, characterized by mass spectrometry, in moderate yield (58%). Bis(bromomethyl)terphenyl **10** was coupled with *p-tert*-butylcalix[4]arene (**14**) to give calixspheranddiol **15** (Chart 3). The ^1H NMR spectrum of **15** shows characteristic signals for the *tert*-butyl groups at δ 1.35, 1.29, and 0.85 in the ratio of 1:1:2, three AB systems at δ 5.46 and 4.29, δ 4.87 and 3.36, and δ 4.25 and 3.16 for the benzylic protons, and a triplet at δ 0.14 for the methyl group of the ethyl aryl ether. The cone conformation of the calix[4]arene moiety was confirmed by the ^{13}C NMR spectrum which showed only one signal for the methylene carbons at δ 30.7.[23] Alkylation of **15** with CH_3I in THF and KO-*t*-Bu as the base afforded calixspherand **16** as the potassium complex in nearly quantitative yield. The potassium complex was identified by its ^1H NMR spectrum. Characteristic high field signals at δ –0.02 for the inwardly rotated methoxy group on the calix[4]arene moiety and at δ –0.54 for the methyl group of the ethyl aryl ether were observed in the ^1H NMR spectrum. The carboxylic acid **17** was then obtained from ester **16** by basic hydrolysis with K_2CO_3 in a mixture of MeOH and H_2O in 95% yield. The formation of **17** was proven by its ^1H and ^{13}C NMR spectra which lack the signals for the ethyl ester group originally present in **16**.

Peptide Coupling. The free ligand of **17** was used for the peptide coupling and was obtained from the corresponding potassium complex by heating in a mixture of

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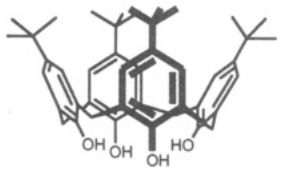
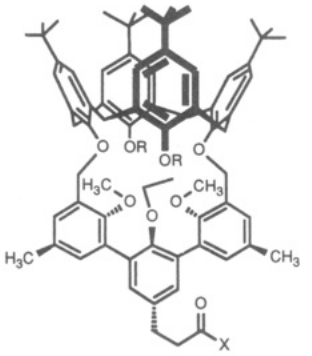
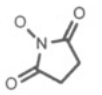
Chart 3



14



15 R = H X = OEt
16 R = Me X = OEt
17 R = Me X = OH
18 R = Me X = 

methanol and water (1:4) under reflux for 3 days. The carboxylic acid function of the free ligands of **17** was activated as its *N*-hydroxysuccinimide ester[16] because it is known that the hydrolysis of *N*-hydroxysuccinimide esters is very slow compared to aminolysis,[24] and therefore these esters are well suited for the formation of stable amide bonds[15] with the free amino groups of the LMWPs. Furthermore, *N*-hydroxysuccinimide esters of drugs and drug derivatives have been previously used with success for the formation of drug–protein conjugates.[12] *N*-Hydroxysuccinimide ester **18** (Chart 3) was obtained by treatment of carboxylic acid **17** with dicyclohexylcarbodiimide (DCC) followed by *N*-hydroxysuccinimide at room temperature in dichloromethane.

Optimal conditions to couple **18** with the LMWP lysozyme were found by varying the solvent system and the molar ratios of reactants. Two solvent systems were tested: dioxane/borate buffer at varying ratios and DMSO with triethylamine. Furthermore, the molar ratio of calixspherand and lysozyme was varied between 0.4 and 2.

The yield of coupled calixspherand, after hydrolysis at room temperature and at 80 °C, was calculated on the basis of total recovered calixspherand. The total amount of recovered calixspherand was determined from the experiment at 80 °C and unbound calixspherand from the hydrolysis at room temperature. The difference being the coupled amount of calixspherand. The best solvent system for the coupling proved to be dioxane/borate buffer (3:2) at pH = 8.5. Similar results were found in DMSO with triethylamine although the reaction time in the latter case is much longer. In both solvent systems, 81–87% of the calixspherand was coupled to lysozyme.[25] When the ratio calixspherand/lysozyme was increased from ~0.4 to ~1.6, the number of calixspherands coupled to lysozyme increased from ~0.3 to ~1.3.

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(25) For a corresponding calixspherand in which the *N*-hydroxysuccinimide ester function is directly attached to the terphenyl unit, the yield of coupled calixspherand was low and not reproducible (0–29% of the added amount).

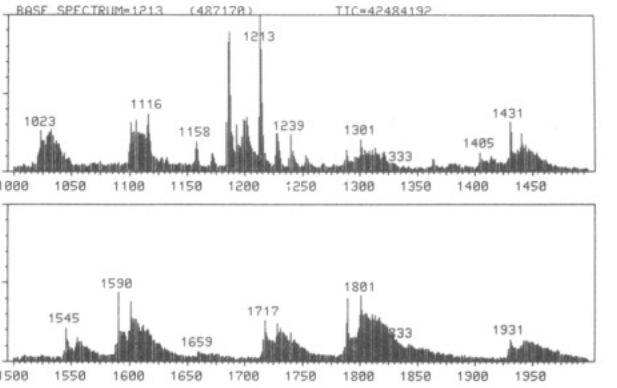

Figure 1. Part of the mass spectrum of the conjugate of lysozyme with $[17\cdot Na]^+$.

Ion-spray mass spectrometry[26] confirmed the formation of a conjugate between lysozyme (lyso) and $[17\cdot Na]^+$ (the Na^+ complex is formed from the free-ligand during the coupling) by the presence of signals at 1545 $\{lyso + [17\cdot Na] + 9H\}^{10+}$, 1717 $\{lyso + [17\cdot Na] + 8H\}^{9+}$, and 1931 $\{lyso + [17\cdot Na] + 7H\}^{8+}$, besides the signals for lysozyme at 1431 $\{lyso + 10H\}^{10+}$, 1590 $\{lyso + 9H\}^{9+}$, and 1789 $\{lyso + 8H\}^{8+}$ (Figure 1).

The research described in this paper shows that it is possible to couple functionalized calixspherands to a low molecular weight protein in good yields. The behavior of the conjugates *in vivo* is currently under investigation.

Experimental Section

Melting points are uncorrected. ^1H NMR spectra (250 MHz) were recorded in deuteriochloroform ($CDCl_3$) with tetramethylsilane (Me_4Si) as an internal standard. Positive ion fast atom bombardment (FAB) mass spectra were obtained with 3-nitrobenzyl alcohol as a matrix. THF was freshly distilled from sodium benzophenone ketyl while dry diethyl ether (Et_2O) was obtained by distillation from lithium aluminum hydride ($LiAlH_4$) and acetonitrile was dried over molecular sieves (3 Å). Hexanes refer to petroleum ether with bp 60–80 °C. NaH was an 80% dispersion in mineral oil and used as such unless otherwise stated. *n*-BuLi was used as a commercially available solution in *n*-hexane (1.6 M). Other chemicals were of reagent grade and were used without purification. Column chromatography was performed with silica gel 60 (SiO_2, E. Merck, particle size 0.040–0.063 mm, 230–240 mesh). All reactions were carried out in a nitrogen atmosphere. Standard workup means that the organic layers were finally washed with brine, dried over magnesium sulfate ($MgSO_4$), filtered, and concentrated *in vacuo*.

The full systematic name for calixspherand is 22*H*,34*H*-4,24-(methano[1,3]benzenomethano)-7,11:12,16:17,21:29,33-tetrametheno-6*H*,28*H*-dibenzo[*b*,*k*][1,13]dioxacyclotriacontin. For convenience, however, the common name, *i.e.*, calixspherand, has been used throughout this paper. The numbering for the calixspherand and the *m*-terphenyl is shown in Chart 4. *p*-*Tert*-butylcalix[4]arene (**14**)[27] and compound **6**[18] were prepared according to literature procedures.

3-(3,5-Dibromo-4-hydroxyphenyl)propionic Acid (2). A solution of Br_2 (17 mL, 0.33 mol) in CH_2Cl_2 (50 mL) was added slowly to a solution of 3-(4-hydroxyphenyl)propionic acid (25 g, 0.15 mol) in CH_2Cl_2 (350 mL). After the mixture was stirred for 1 h, 5% aqueous $NaHSO_3$ (150 mL) was added. The organic layer was separated and washed with H_2O (150 mL), followed by standard workup. The residue was crystallized from 1:1 *i*-Pr_2O/hexanes to afford **2** as white crystals: yield 88%. The product was identical to previously reported material, mp 108–111 °C (lit.[17] mp 107–108 °C).

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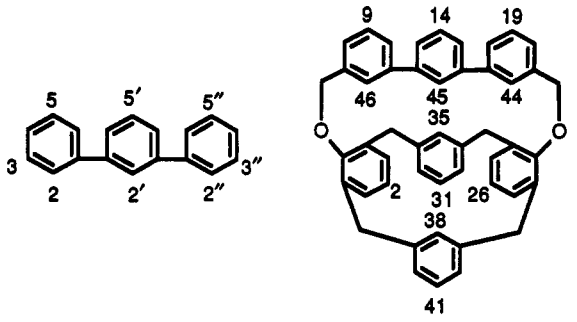
Chart 4



Terphenyl Calixspherand

3-(3,5-Dibromo-4-ethoxyphenyl)propionic Acid Ethyl Ester (3). A mixture of **2** (4.90 g, 15 mmol), Et_2SO_4 (4.6 mL, 35 mmol), and K_2CO_3 (4.80 g, 35 mmol) in CH_3CN (100 mL) was heated under reflux overnight. After the solution was cooled to rt, the solvent was removed *in vacuo* and the residue dissolved in EtOAc (100 mL) and washed with water (100 mL) followed by standard workup. The crude product was purified by Kugelrohr distillation (o.t. 150 °C, 8×10^{-2} mbar) to afford **3** as a colorless liquid: yield 89%; n^{20}_D = 1.5452; ^{1}H NMR δ 7.34 (s, 2 H), 4.14 (q, 2 H, J = 7.1 Hz), 4.05 (q, 2 H, J = 7.0 Hz), 2.86 (t, 2 H, J = 7.6 Hz), 2.58 (t, 2 H, J = 7.6 Hz), 1.47 (t, 3 H, J = 7.0 Hz), 1.24 (t, 3 H, J = 7.6 Hz); ^{13}C NMR δ 172.2, 151.8, 139.0 (s), 132.4 (d), 118.2 (s), 69.4, 60.6, 35.4, 29.6 (t), 15.4, 14.2 (q); IR (KBr) 1734 (C=O) cm^{-1}; mass spectrum (EI), m/e 377.945 (M^+, calcd 377.947). Anal. Calcd for $C_{13}H_{16}Br_2O_3$: C, 41.08; H, 4.24. Found: C, 41.36; H, 4.41.

1-Bromo-2-methoxy-3-(methoxymethyl)-5-methylbenzene (4). To a solution of sodium methoxide in methanol, prepared from sodium (9 g, 0.39 mol) and methanol (500 mL), was slowly added a mixture of 3-(chloromethyl)- and 3-(bromomethyl)-1-bromo-2-methoxy-5-methylbenzene (65 g).[18] The solution was heated under reflux for 1 h, cooled to rt, and evaporated. The residue was dissolved in EtOAc (500 mL) and washed with water (200 mL) followed by standard workup. The residue was distilled to afford **4** as a colorless liquid (bp 83–93 °C/5 × 10^{-2} Torr) which was identical to previously reported material.[8] Yield: >88%.

(2-Methoxy-3-(methoxymethyl)-5-methylphenyl)boronic Acid (5). To a solution of **4** (24.5 g, 0.1 mol) in THF (250 mL) was added *n*-BuLi (70 mL, 0.11 mol) at −78 °C. After the solution was stirred for 6 min, $B(OMe)_3$ (70 mL, 0.6 mol) was added. The mixture was stirred for 30 min at −78 °C, warmed to 0 °C over 45 min, diluted with 2 M aqueous HCl (150 mL), and stirred for 30 min at rt. Ether (300 mL) was then added, the mixture was stirred for 30 min at rt, and the layers were separated. The aqueous layer was extracted with ether (2 × 100 mL). The combined ether layers were extracted with 3 M aqueous NaOH (4 × 100 mL). The basic extracts were cooled to 5 °C, acidified to pH 1 with concentrated HCl, and extracted with ether (4 × 100 mL) followed by standard workup to afford **5** as a clear brown oil in 83% yield, which was identical to previously reported material.[8] Compound **5** was stored in the refrigerator and used without further purification.

1-Bromo-2-methoxy-3-((methoxymethoxy)methyl)-5-methylbenzene (7). A mixture of **6** (11.33 g, 49 mmol) and NaH (60% in oil, 2.2 g, 55 mmol) in THF (125 mL) was heated under reflux for 1 h. After being cooled to rt, a solution of chloromethyl methyl ether (5.1 mL, 50 mmol) in THF (25 mL) was added dropwise and the mixture stirred for 0.5 h at rt. Subsequently the mixture was heated under reflux for 1 h. After being cooled to rt, the mixture was poured into water (250 mL) and extracted with ether (2 × 150 mL), followed by standard workup. The residue was purified by Kugelrohr distillation (o.t. 100 °C/3 × 10^{-2} mbar) to afford **7** as a colorless liquid: yield 88%; n^{20}_D = 1.5272; ^{1}H NMR δ 7.32 (d, 1 H, J = 1.8 Hz), 7.16 (d, 1 H, J = 1.6 Hz), 4.73 (s, 2 H), 4.62 (s, 2 H), 3.83 (s, 3 H), 3.43 (s, 3 H), 2.29 (s, 3 H); ^{13}C NMR δ 153.0, 116.9 (s), 96.0, 64.5 (t), 61.5, 55.4, 20.5 (q); mass spectrum (EI), m/e 274.020 (M^+, calcd for $C_{11}H_{15}BrO_3$ 274.021).

(2-Methoxy-3-((methoxymethoxy)methyl)-5-methylphenyl)boronic Acid (8). To a solution of **7** (5.50 g, 20 mmol) in THF (100 mL) was added a solution of *n*-BuLi (15 mL, 24 mmol) at −78 °C. The reaction mixture was stirred for an additional 15 min at −78 °C, treated with $B(OMe)_3$ (6.8 mL, 60 mmol), allowed to warm to rt over 2 h, and acidified to pH ~6.5 with 1 M aqueous HCl. The reaction mixture was extracted with CH_2Cl_2 (2 × 50 mL) followed by standard workup to afford **8** in 83% yield as an oil which was stored in the refrigerator and used without further purification.

Tetrahydro-2-[3-((methoxymethoxy)methyl)-2-methoxy-5-methylphenyl]-4*H*-1,3,6,2-dioxazaborocine (13). A solution of boronic acid **8** (0.25 g, 1.04 mmol) and diethanolamine (0.12 mL, 1.14 mmol) in toluene (10 mL) was heated under reflux for 5 min. The toluene was removed *in vacuo*, and the resulting solid was washed with ether and recrystallized from EtOAc to afford **13** as white crystals: yield 38%; mp 157–159 °C (EtOAc); ^{1}H NMR δ 7.40 (d, 1 H, J = 2.0 Hz), 7.11 (d, 1 H, J = 2.0 Hz), 5.58 (br s, 1 H), 4.72 (s, 2 H), 4.58 (s, 2 H), 4.11–4.01 (m, 2 H), 3.89–3.81 (m, 2 H), 3.81 (s, 3 H), 3.43 (s, 3 H), 3.43–3.34 (m, 2 H), 2.85–2.75 (m, 2 H), 2.29 (s, 3 H); ^{13}C NMR δ 159.6 (s), 95.8, 64.5 (t), 63.4 (q), 62.8 (t), 55.4 (q), 52.0 (t), 20.9 (q); mass spectrum (EI), m/e 309.172 (M^+, calcd 309.175). Anal. Calcd for $C_{15}H_{24}BNO_5$: C, 58.27; H, 7.82; N, 4.53. Found: C, 57.92; H, 7.74; N, 4.51.

3-[2'-Ethoxy-2,2''-dimethoxy-3,3''-bis((methoxymethoxy)methyl)-5,5''-dimethyl-1,1':3',1''-terphenyl-5'-yl]propionic Acid Ethyl Ester (11). To a vigorously stirred mixture of boronic acid **8** (4.08 g, 17 mmol) and **3** (2.66 g, 7 mmol) in a mixture of benzene (60 mL), ethanol (15 mL), and 2 M aqueous Na_2CO_3 (30 mL) was added $Pd(PPh_3)_4$ (0.24 g, 0.2 mmol). The mixture was heated under reflux for 40 h (70 mg of fresh catalyst was added after 20 h). After the solution was cooled to rt, the layers were separated, and the organic layer was dried and concentrated *in vacuo*. Compound **11** was not purified but directly converted to **12**.

3-[2'-Ethoxy-3,3''-bis(hydroxymethyl)-2,2''-dimethoxy-5,5''-dimethyl-1,1':3',1''-terphenyl-5'-yl]propionic Acid Ethyl Ester (12). To a solution of crude **11** in methanol (40 mL) was added concentrated aqueous HCl (0.5 mL). The mixture was heated under reflux for 15 min, cooled to rt, and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2, EtOAc/hexanes 1:3) to afford **12** as a colorless oil: yield, 50% over two steps; ^{1}H NMR δ 7.20 (s, 2 H), 7.16 (br s, 2 H), 7.12 (br s, 2 H), 4.74 (br s, 4 H), 4.12 (q, 2 H, J = 7.1 Hz), 3.47 (s, 6 H), 3.38 (q, 2 H, J = 7.0 Hz), 2.97 (t, 2 H, J = 7.7 Hz), 2.65 (t, 2 H, J = 7.7 Hz), 2.39 (br s, 2 H), 2.34 (s, 6 H), 1.23 (t, 3 H, J = 7.1 Hz), 0.74 (t, 3 H, J = 7.0 Hz); ^{13}C NMR δ 172.8, 153.7, 153.2 (s), 68.8, 61.8 (t), 60.8 (q), 60.4, 36.2, 30.3 (t, CH_2CO_2), 20.8, 15.4, 14.2 (q); IR (KBr) 3432 (OH), 1734 (C=O) cm^{-1}; mass spectrum (EI), m/e 522.264 (M^+, calcd for $C_{31}H_{38}O_7$ 522.262).

3-[3,3''-Bis(bromomethyl)-2'-ethoxy-2,2''-dimethoxy-5,5''-dimethyl-1,1':3',1''-terphenyl-5'-yl]propionic Acid Ethyl Ester (10). To a solution of **12** (1.83 g, 3.5 mmol) in benzene (75 mL) was slowly added PBr_3 (0.5 mL, 5 mmol). The mixture was stirred at rt for 20 min, whereupon water (60 mL) and saturated $NaHCO_3$ (15 mL) were added successively. The benzene was removed *in vacuo* and the remaining aqueous solution was extracted with CH_2Cl_2 (2 × 50 mL), followed by standard workup. The residue was purified by flash chromatography (SiO_2, CH_2Cl_2) to afford **10** as an oil: yield 58%; ^{1}H NMR δ 7.21 (s, 2 H), 7.19 (d, 2 H, J = 2.0 Hz), 7.14 (d, 2 H, J = 2.0 Hz), 4.63 (s, 4 H), 4.12 (q, 2 H, J = 7.1 Hz), 3.53 (s, 6 H), 3.36 (q, 2 H, J = 7.0 Hz), 2.98 (t, 2 H, J = 7.7 Hz), 2.65 (t, 2 H, J = 7.7 Hz), 2.33 (s, 6 H), 1.24 (t, 3 H, J = 7.1 Hz), 0.74 (t, 3 H, J = 7.0 Hz); ^{13}C NMR δ 172.8, 154.0, 153.1 (s), 68.9 (t), 60.8 (q), 60.5, 36.2, 30.3, 29.0 (t), 20.7, 15.4, 14.3 (q); IR (KBr) 1734 (C=O) cm^{-1}; mass spectrum (EI), m/e 646.096 (M^+, calcd for $C_{28}H_{32}O_6$ 646.093).

3-[2,26,31,41-Tetrakis(1,1-dimethylethyl)-45-ethoxy-35,38-dihydroxy-44,46-dimethoxy-9,19-dimethylcalixspherand-14-yl]propionic Acid Ethyl Ester (15). A mixture of *p-tert*-butylcalix[4]arene **14** (1.34 g, 1.8 mmol), NaH (60% in oil, 0.36 g, 9 mmol), and 3 mol % of 18-crown-6 in THF (300 mL) was stirred at rt until no more hydrogen gas evolved (ca. 0.5 h). The mixture was then heated under reflux and a solution of terphenyl **10** (1.18 g, 1.8 mmol) in THF (50 mL) was added dropwise. The

resulting mixture was further heated under reflux for 3 h and cooled to rt. H_2O (5 mL) was added, the THF was removed *in vacuo*, and the residue was partitioned between CH_2Cl_2 (100 mL) and 1 M HCl (50 mL), followed by standard workup. The residue was purified by flash chromatography (SiO_2, EtOAc:hexanes 1:4) followed by recrystallization from $CHCl_3$/MeOH or 2-propanol to afford **15** as a white solid: yield 69%; mp >250 °C dec (*i*-PrOH); ^{1}H NMR δ 7.34 (s, 2 H), 7.11 (s, 2 H), 7.03 (d, 2 H, J = 1.7 Hz), 6.98 (s, 2 H), 6.97 (br s, 2 H), 6.73 (s, 1 H), 6.69 (d, 2 H, J = 2.3 Hz), 6.57 (d, 2 H, J = 2.3 Hz), 5.74 (s, 1 H), 5.46 and 4.29 (ABq, 4 H, J = 9.9 Hz), 4.87 and 3.36 (ABq, 4 H, J = 12.8 Hz), 4.25 and 3.16 (ABq, 4 H, J = 13.4 Hz), 4.14 (q, 2 H, J = 7.2 Hz), 3.48 (s, 6 H), 3.09 (t, 2 H, J = 7.7 Hz), 2.81–2.72 (m, 4 H), 2.30 (s, 6 H), 1.35, 1.29 (s, 2 × 9 H), 1.26 (t, 3 H, J = 7.2 Hz), 0.85 (s, 18 H), 0.14 (t, 3 H, J = 7.0 Hz); ^{13}C NMR δ 172.9, 158.6, 155.2, 151.8, 151.1, 151.0, 145.8, 140.2, 139.9 (s), 76.5, 68.3 (t), 60.3 (q), 36.4 (t), 33.8, 33.7 (s), 31.9, 31.7, 31.0 (q), 30.7, 30.6 (t), 20.5, 14.5, 14.3 (q); IR (KBr) 3497 (OH), 1736 (C=O) cm^{-1}; mass spectrum (FAB), *m/e* 1157.7 ((M + Na)$^+$, calcd 1157.7). Anal. Calcd for $C_{75}H_{90}O_9 \cdot 0.4CHCl_3$: C, 76.54; H, 7.70. Found: C, 76.14; H, 7.56. The presence of $CHCl_3$ was confirmed by ^{1}H NMR in CD_2Cl_2.

3-[2,26,31,41-Tetrakis(1,1-dimethylethyl)-45-ethoxy-35,38,-44,46-tetramethoxy-9,19-dimethylcalixspherand-14-yl]propionic Acid Ethyl Ester (16). A solution of KO-*t*-Bu (0.24 g, 2.1 mmol) in THF (30 mL) was added slowly to a solution of **15** (0.79 g, 0.7 mmol) and MeI (0.18 mL, 2.8 mmol) in THF (50 mL). After the addition of KO-*t*-Bu, another 6 equiv of MeI (0.26 mL, 4.2 mmol) was added and the resulting solution stirred for 1 h at 40 °C. Subsequently 1 M HCl (5 mL) was added and the THF removed *in vacuo*. The residue was redissolved in CH_2Cl_2 (100 mL) and the organic solution washed with 1 M HCl (50 mL) and saturated Na_2SO_3 (30 mL), followed by standard workup. The residue was redissolved in MeOH. Undissolved material was removed by filtration and the filtrate evaporated to dryness to yield **16** as a potassium iodide complex. For analytical purposes a small sample was converted to the potassium picrate complex by anion exchange. **Potassium picrate complex:** ^{1}H NMR δ 8.77 (s, 2 H), 7.41 (s, 2 H), 7.36 (s, 2 H), 7.24 (d, 2 H, J = 2.3 Hz), 7.17 (d, 2 H, J = 1.5 Hz), 7.05 (br s, 2 H), 6.97 (d, 2 H, J = 2.3 Hz), 6.83 (s, 2 H), 5.74 and 4.14 (ABq, 4 H, J = 10.9 Hz), 4.53 and 3.68 (ABq, 4 H, J = 12.5 Hz), 4.14 (q, 2 H, J = 7.1 Hz), 4.01 (s, 3 H), 3.74 and 3.36 (ABq, 4 H, J = 15.6 Hz), 3.58 (s, 6 H), 3.11 (t, 2 H, J = 7.4 Hz), 2.75 (t, 2 H, J = 7.4 Hz), 2.37 (s, 6 H), 2.22 (q, 2 H, J = 7.1 Hz), 1.30, 1.17 (s, 2 × 9 H), 1.25 (t, 3 H, J = 7.2 Hz), 1.07 (s, 18 H), –0.02 (s, 3 H), –0.54 (t, 3 H, J = 7.1 Hz); ^{13}C NMR δ 172.6, 155.2, 155.1, 154.4, 152.6, 152.4, 148.3, 147.6, 147.3 (s), 75.9, 69.5 (t), 62.9, 62.6, 60.6 (q), 60.4, 36.1, 34.9, 29.9 (t), 34.4, 34.2, 34.1 (s), 31.6, 31.4, 31.1 (q), 30.5 (t), 20.9, 14.3, 13.2 (q); IR (KBr) 1733 (C=O) cm^{-1}; mass spectrum (FAB), *m/e* 1201.6 (M$^+$, calcd for $C_{77}H_{94}KO_9$ 1201.7).

3-[2,26,31,41-Tetrakis(1,1-dimethylethyl)-45-ethoxy-35,38,-44,46-tetramethoxy-9,19-dimethylcalixspherand-14-yl]propionic Acid (17). A mixture of **16** (0.8 g, 0.6 mmol) and K_2CO_3 (0.33 g, 2.4 mmol) in EtOH (30 mL) and water (15 mL) was heated under reflux for 4 h. After being cooled to rt, the solution was acidified to pH 3 with 6 M aqueous HCl and extracted with CH_2Cl_2 (3 × 25 mL) followed by standard workup to afford **17** as a white solid in 95% yield. **Potassium picrate complex:** mp >190 °C dec (CH_2Cl_2/*i*-Pr_2O); ^{1}H NMR δ 8.75 (s, 2 H), 7.49 (s, 2 H), 7.36 (s, 2 H), 7.24 (d, 2 H, J = 2.2 Hz), 7.17 (br s, 2 H), 7.00 (br s, 2 H), 6.96 (d, 2 H, J = 2.1 Hz), 6.83 (s, 2 H), 5.74 and 4.09 (ABq, 4 H, J = 10.7 Hz), 4.55 and 3.68 (ABq, 4 H, J = 12.6 Hz), 4.03 (s, 3 H), 3.78 and 3.34 (ABq, 4 H, J = 15.4 Hz), 3.61 (s, 6 H), 3.10 (t, 2 H, J = 7.4 Hz), 2.90 (t, 2 H, J = 7.4 Hz), 2.36 (s, 6 H), 2.29 (q, 2 H, J = 7.1 Hz), 1.31, 1.19 (s, 2 × 9 H), 1.08 (s, 18 H), –0.02 (s, 3 H), –0.54 (t, 3 H, J = 7.1 Hz); ^{13}C NMR δ 162.4, 155.3, 154.5, 152.6, 152.4, 148.1, 147.5, 147.2 (s), 76.0, 69.5 (t), 63.2. 60.4 (q), 36.1, 34.9, 29.9 (t), 34.4, 34.2, 34.1 (s), 31.6, 31.4 31.1 (q), 30.5 (t), 20.9, 13.1 (q); IR (KBr) 1731 (C=O) cm^{-1}; mass spectrum (FAB), *m/e* 1173.6 (M$^+$, calcd for $C_{75}H_{90}KO_9$ 1173.6).

Free Ligand. A mixture of the potassium complex in an ethanol/water mixture (1:4) was heated under reflux for 3 days. After being cooled to rt, the mixture was extracted with CH_2Cl_2 followed by standard workup to afford the free ligand as a white solid: mp >220 °C dec (MeOH/CH_2Cl_2); ^{1}H NMR δ 7.31 (s, 2 H), 7.18 (s, 2 H), 7.10 (br s, 2 H), 7.01 (br s, 2 H), 6.93 (s, 2 H), 6.67 (br s, 2 H), 6.52 (br s, 2 H), 5.51 and 4.17 (ABq, 4 H, J = 10.2 Hz), 4.57 and 3.29 (ABq, 4 H, J = 12.3 Hz), 4.09 (d, 2 H, J = 12.9 Hz), 3.72 (br s, 3 H), 3.44 (s, 6 H), 3.07–2.96 (m, 9 H), 2.76 (t, 2 H, J = 7.4 Hz), 2.33 (s, 6 H), 1.35, 1.26 (s, 2 × 9 H), 0.85 (s, 18 H), 0.22 (t, 3 H, J = 7.1 Hz). Anal. Calcd for $C_{75}H_{90}O_9$: C, 79.33; H, 7.99. Found: C, 78.95; H, 8.21.

1-[1-Oxo-3-[2,26,31,41-tetrakis(1,1-dimethylethyl)-45-ethoxy-35,38,44,46-tetramethoxy-9,19-dimethylcalixspherand-14-yl]propoxy]-2,5-pyrrolidinedione (18). The free ligand of carboxylic acid **17** (0.10 g, 0.09 mmol) was dissolved in CH_2Cl_2 (20 mL) and DCC (50 mg, 0.26 mmol) was added. The solution was stirred for 15 min. Thereafter NHS (30 mg, 0.26 mmol), dried at 50 °C *in vacuo* for 16 h, was added and the resulting mixture was stirred overnight. The turbid solution was subsequently filtered over Celite and evaporated *in vacuo*. The solid material was washed with MeOH and dried to afford **18** as a white solid in a yield of 91% and was used as such: ^{1}H NMR δ 7.32 (s, 2 H), 7.18 (s, 2 H), 7.10 (br s, 2 H), 6.99 (br s, 2 H), 6.92 (s, 2 H), 6.65 (br s, 2 H), 6.50 (br s, 2 H), 5.51 and 4.16 (ABq, 4 H, J = 10.2 Hz), 4.57 and 3.29 (ABq, 4 H, J = 12.3 Hz), 4.08 (d, 2 H, J = 13.0 Hz), 3.72 (br s, 3 H), 3.43 (s, 6 H), 3.04–2.74 (m, 12 H), 2.32 (s, 6 H), 2.00–1.62 (m, 4 H), 1.34, 1.25 (s, 2 × 9 H), 0.84 (s, 18 H), 0.21 (t, 3 H, J = 7.1 Hz).

Lysozyme Conjugates. Method A. A solution of 2,5-pyrrolidinedione **18** in 1,4-dioxane (0.65 mL) was slowly added to a solution of lysozyme in borate buffer (0.4 mL, 25 mM; pH 8.5) at rt and stirred for 6 h. The solution was then lyophilized and analyzed.

Method B. To a solution of lysozyme in DMSO (0.6 mL) at rt was slowly added a solution of 2,5-pyrrolidinedione **18** in DMSO (0.45 mL) followed by Et_3N (7.5 μL). The resulting mixture was stirred for another 65 h. The solution was then lyophilized and analyzed.

Characterization of Calixspherand–Lysozyme Conjugates. The residue obtained after lyophilization was dissolved in DMSO (400 μL) and two 50-μL aliquots were taken. To both samples was added 6 M aqueous NaOH (50 μL); one sample was heated at 80 °C and the other sample was kept at rt. After 70 h, $CHCl_3$ (200 μL) was added to both samples and the samples were vortexed for 1 min. The layers were than separated and the amount of calixspherand in the organic layer was determined by HPLC (*vide infra*). It was assumed that no hydrolysis occurred at rt, and free calixspherand present in the solution that was hydrolyzed at rt therefore represents unbound calixspherand. The amount of calixspherand in the sample which was hydrolyzed at 80 °C was taken as total recovered calixspherand. Calibration curves were constructed by hydrolysis of known amounts of calixspherand under identical conditions.

HPLC Analysis of calixspherands was performed with 20-μL samples on a μ-Bondapack C-18 column (30 cm). A mixture of MeOH, water, and a solution of 250 g/L of NH_4OAc in MeOH in a ratio of 1000:15:2 was used as eluent at a flow rate of 1 mL/min. Detection was performed by UV at 250 nm.

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Supplementary Material Available: ^{1}H NMR spectra of compounds **7**, **10**, **12**, **16**, and [**17**·K]$^+$ (5 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.