Synthesis of Fluorescent Derivatives of the Antibiotic Moenomycin A

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Dedicated to Professor Lutz Tietze on the occasion of his 60th birthday

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Moenomycin has been highly selectively converted into amino derivative **3b**. The primary amino group of this compound can be used to attach various fluorescent labels to moenomycin, through conversion of isothiocyanates into thioureas and squaric acid diesters into diamides. The application of some of the derivatives is outlined.
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Introduction

Antibiotic resistance in bacterial pathogens has become a serious problem for human health and requires both the development of antiinfectives with novel modes of action and a deeper mechanistic understanding of already existing drugs.[1]

The shape of a bacterium is determined by a net-like multilayer polymer surrounding the cell. This polymer − peptidoglycan − consists of repeating β-1,4-linked *N*-acetylglucosaminyl-*N*-acetylmuramyl units cross-linked through short peptide side chains.[2] The biosynthesis of peptidoglycan is an essential pathway for bacteria and has no direct counterpart in eukaryotic cells. Defects or disruption of peptidoglycan or inhibition of its biosynthesis result in cell lysis caused by the osmotic pressure. The distinct stages of peptidoglycan biosynthesis thus offer attractive targets for the development of selective antibacterial agents.

E. coli peptidoglycan biosynthesis, starting from a membrane-bound undecaprenyl-linked disaccharide precursor (lipid II), is completed by two successive reactions: a transglycosylation reaction producing unbranched glycan strands[2,3] and a transpeptidation reaction cross-linking the peptide units of different strands.[2] Both reactions are catalyzed by the major high molecular weight penicillin-binding proteins (PBPs).[4] PBPs such as PBP 1a and 1b are bifunctional enzymes with two separate active sites: one for transglycosylation and the other for transpeptidation. Each of these domains can be specifically inhibited by antibiotics. While β-lactam antibiotics exert their action through covalent binding to an essential serine residue in the transpeptidase domain, the transglycosylation step of cell wall assembly can be blocked by a number of antibiotics, including the moenomycins.[5] Of these antibiotics, however, the moenomycins (see moenomycin A, **1**) are the only compounds known to inhibit the enzyme (Scheme 1).[3] The structure-activity relationships of the moenomycins have been studied extensively[6] and a mechanism for their mode of action has been proposed.[7,8] It is assumed that they are anchored to the cytoplasmic membrane through the moenocinol lipid component, followed by highly selective recognition of the oligosaccharide moiety at a substrate binding site of the enzyme, most probably the binding site of the growing glycan chain (the glycosyl donor). Whereas the mechanism of the transpeptidation reaction is reasonably well understood, the active site of the transglycosylase is still unknown, and the mechanism of the transglycosylation step is largely unexplored. The moenomycins are a unique tool for elucidation of the structure of the enzyme and the detailed mechanism of the transglycosylation reaction.

Research procedures based on fluorescence methods are of great importance in biochemistry.[9] In the course of investigations into moenomycin we had already made use of fluorescence phenomena in studying the interaction of moenomycin with artificial membranes[10] and with moenomycin-binding aptamers selected by an in vitro selection/amplification procedure from a library of approximately 7×10^{14} different modified RNA sequences.[11]

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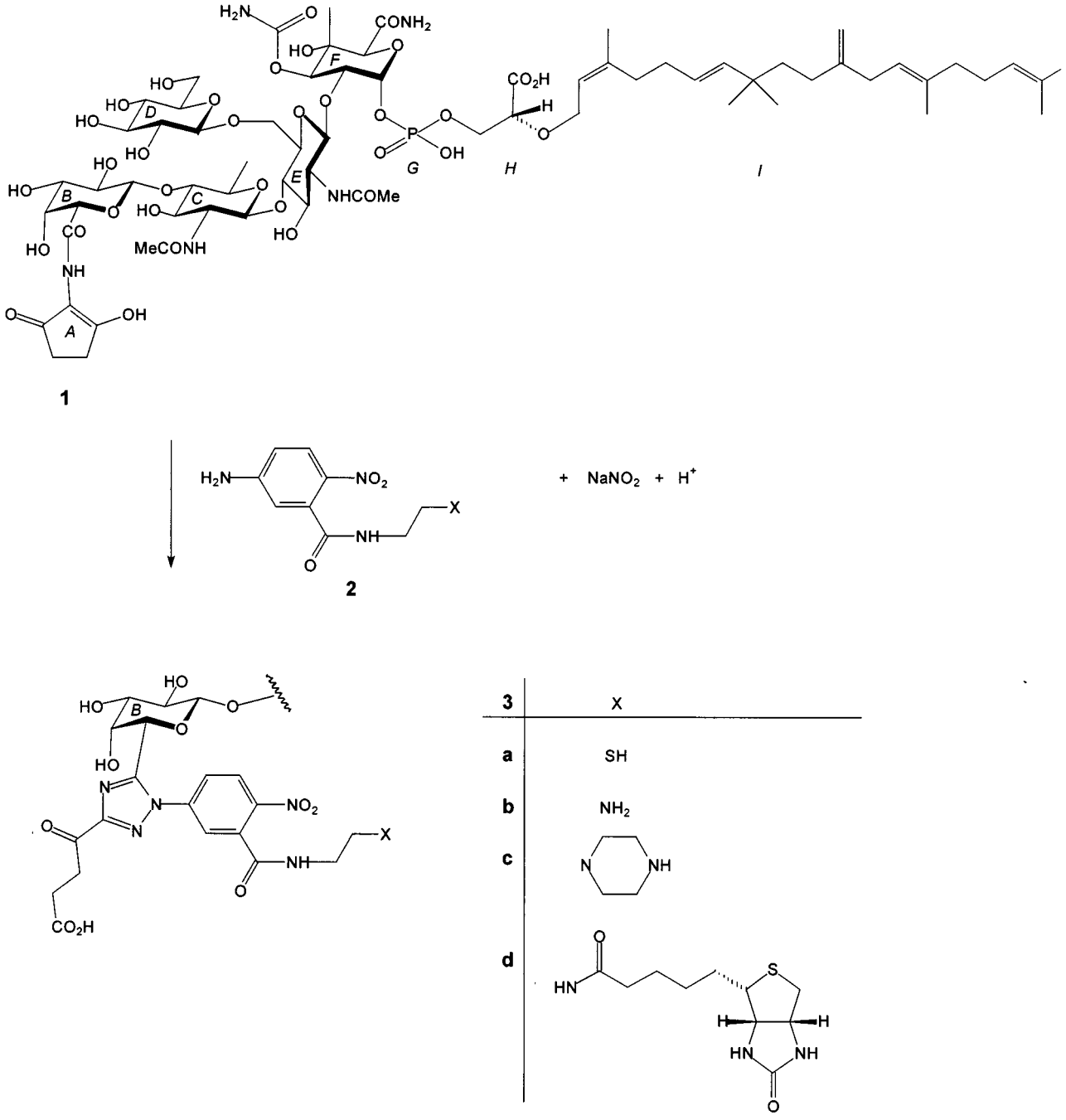


Scheme 1

This publication describes the chemistry that can be used to attach chromophores to suitable moenomycin derivatives.

Results and Discussion

Treatment of Moenomycin A with Aromatic Diazonium Salts

We have previously shown that **1** reacts selectively with diazonium salts at the enolized β-diketone unit A.[12] The primary coupling product undergoes a Japp−Klingemann cleavage[13] and the thus formed amidrazone cyclizes to give a triazole, the final product of the reaction. The reaction occurs at a site that has been found to be of minor importance for the antibiotic activity.[6] By this sequence, the thiol-substituted compound **3a** was prepared and used for the synthesis of a number of bioconjugates by 1,4-addition to maleimides[12] and by disulfide formation .[14] Recently, we reported that the amino compound **3b** is also accessible by this route.[15,16] The heterobifunctional reagent **2** (X = NH_2) was prepared from 5-amino-2-nitrobenzoic acid by Staab's procedure.[17,18] The selective formation of the aromatic diazonium salt from **2** (X = NH_2) is the consequence

of the different p*K* values of the amino groups. Under the conditions of diazonium salt formation, the aliphatic amino group is protected by protonation. Compound **3b** was obtained in a yield of 78% after careful purification.[19]

The formation of moenomycin conjugates by the Japp−Klingemann route seems to be general and can be used for the synthesis of other conjugates as well. Thus, under similar conditions the piperazine derivative **3c** and the biotin conjugate **3d** were prepared. Compound **3d** has been employed for the isolation of moenomycin binding aptamers.[11] Amino compound **3b** has turned out to be a much more flexible ligand than thiol **3a**, since more coupling reactions are available for the preparation of bioconjugates; they include treatment with activated carboxylic acids to provide amides,[16] addition to isothiocyanates to give thioureas, and the attachment to other amines through the bifunctional squaric acid linker[20,21] as detailed below.

Synthesis of Coumarin Conjugate 6a

The synthesis of the coumarin-derived isothiocyanate **5c** started from coumarin **4**, the double bond of which was arylated in the 3-position with the diazonium salt prepared from *N*-acetyl-*p*-phenylenediamine by Meerwein's arylation reaction (Scheme 2).[22] Under typical conditions[22] (not optimized), **5a** was obtained in acceptable yield. The absence of the coumarin 3-H signal in the ^{1}H NMR spectrum of **5a** and the long-range coupling of 2′-H, 6′-H, and the CH_3-9 protons to C-3 (HMBC) confirmed the proposed structure. FAB MS displayed the correct molecular ions. Heating of compound **5a** under reflux in 6% hydrochloric acid in dry ethanol brought about selective removal of the *N*-acetyl group. The required amine **5b** was obtained in 93% yield after FC.

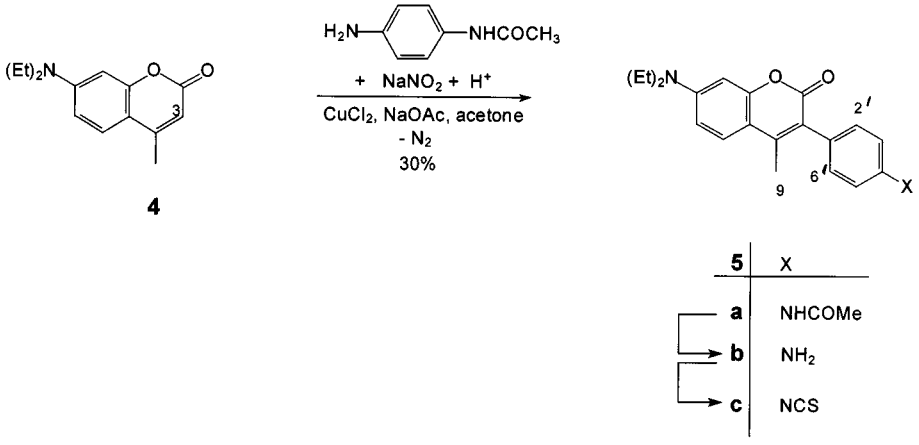
The preparation of isothiocyanates from aromatic amines and thiophosgene can be performed under different conditions: (i) in a heterogeneous mixture of water and chloroform[23] with calcium carbonate as the base,[24] (ii) in homogeneous organic solutions,[25] or (iii) in water or aqueous hydrochloric acid.[26] A heterogeneous mixture of water/chloroform and calcium carbonate was used for the preparation of **5c** (54% yield after FC). The IR spectrum of **5c** showed the strong absorption at 2113 cm^{-1} characteristic of isothiocyanates. Furthermore, two signals appeared in the ^{13}C NMR spectrum, at δ = 134.79 and 136.01, corresponding to C-4′ and the isothiocyanato group. The FAB MS exhibited the expected molecular ion peak.

Coupling between **5c** and **3b** was performed in a mixture of DMF and pyridine[27] (to make the reaction solution homogeneous) at room temperature under argon and in the dark. The reaction mixture was stirred until **3b** had been completely consumed (TLC analysis). In the ^{1}H and ^{13}C NMR spectra of the reaction product **6a**, all signals of the coumarin moiety (Cou), the nitroaromatic ring and the ethylenediamine-derived components (Ar and DAE), the triazole ring (TA), the lipid component (I), the carboxypropionyl appendage (A), and the thioureido unit [NH*C*(S)NH at δ = 181.50] were assigned with the aid of 2D NMR experiments (Table 1). The carbon signal of C-1^I was covered by sugar carbon signals and the C-2^A carbon signal seemed to be hidden under the solvent (DMSO) signal (HMQC). Some sugar proton and carbon signals were assigned, but most of the sugar part signals could not be identified, due to overlapping and broadening. The high-resolution ESI FT ICR MS spectra in both modes confirmed structure **6a**. The fluorescence spectra of **6a** and **5c** in methanol/water (1:1) solution were practically identical.

With the aid of **6a**, the extent of anchoring of moenomycin to artificial membranes was studied by means of fluorescence methods, to yield partition coefficients. Furthermore, the transfer of **6a** between POPC vesicles was investigated by fluorescence resonance energy transfer (FRET), by making use of (4-dimethylamino)azobenzene, which efficiently quenches the fluorescence of the 7-diethylamino-4-methylcoumarin chromophore. From these experiments a rate constant was calculated.[10,28]

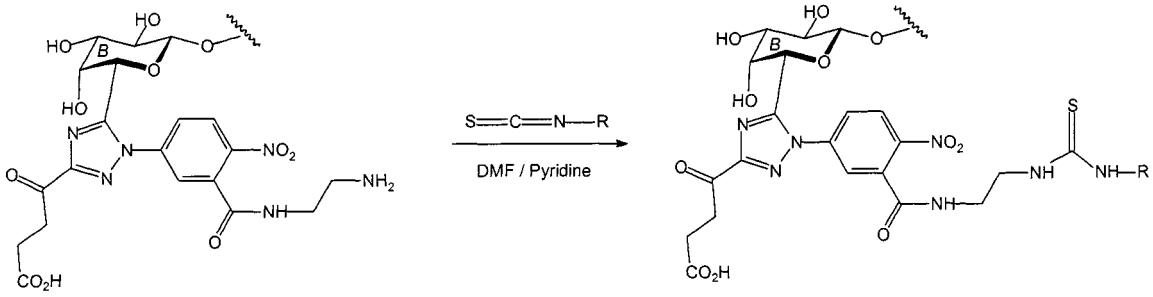
Conjugation of 6-Aminotetramethylrhodamine (7a) to 3b

The long-wavelength rhodamine fluorophores are among the most photostable fluorescent labeling reagents available. Unlike fluorescein, which forms a nonfluorescent spiro form at acidic pH, rhodamines exist in a fluorescent quinone form at neutral and acidic pH values and in a non-

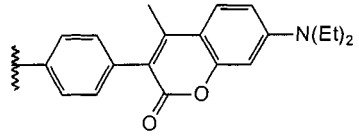
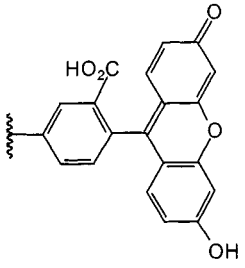
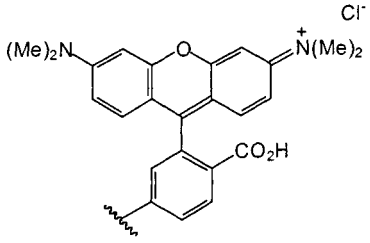
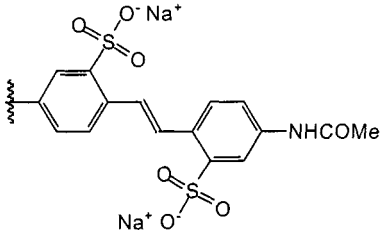


Scheme 2

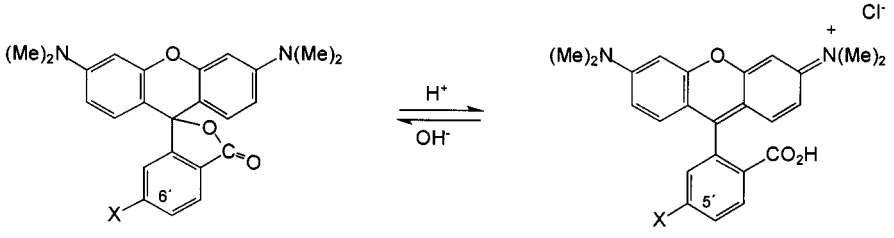
Table 1. Structures and fluorescence data of compounds **6a**−**6d**



3b **6**

6	R	Emission (excitation) λ_{max} [nm]	**6**	R	Emission (excitation) λ_{max} [nm]
a		476 (397)	**c**		521 (492)
b		577 (544)	**d**		412 (346)

fluorescent spiro form at basic pH values (Scheme 3). Tetramethylrhodamine is readily excited by the intense 546 nm spectral line of mercury arc lamps or by the 543 nm spectral line of the green He-Ne laser, which is increasingly being used for analytical purposes such as fluorescence correlation spectroscopy. For conjugation, the rhodamines usually



7	X
a	NH_2
b	NCS

Scheme 3

have a carboxy or an amino (isothiocyanato) group in position(s) 5 and/or 6 (spiro form).[29] The commercially available pure tetramethylrhodamine isothiocyanate isomers (Molecular Probes) were not used because of their high cost, whilst the mixture of the two isomers (Fluka) was found to be of unsatisfactory purity. The same problems with the purity of the commercial tetramethylrhodamines have been reported previously.[30] A synthesis of the isomeric 5- and 6-amines (spiro form numbering) has been described by Corrie and Craik,[30] and **7a** was prepared by this route. The ^{13}C NMR spectra of amine **7a**, with the ^{13}C signal of the spiro carbon atom at $\delta = 83.90$ (C-9′), confirmed the proposed structure. The corresponding isothiocyanate **7b** was prepared as described for **5c**, purified and immediately used for coupling with **3b**.

Moenomycin−tetramethylrhodamine derivative **6b** was characterized, after careful purification, by FAB MS and ESI FT ICR MS. The mass spectra were consistent with structure **6b**. NMR analysis in this case was not successful for two reasons: (i) the signals were broad both in the ^1H and in the ^{13}C spectra, and (ii) only a small amount of **6b** was available. In the fluorescence spectrum of **6b**, a small blue shift (compared with **7a**) in the excitation (−3 nm) and a red shift in emission (+6 nm) maxima were observed.[9] A derivative of **6b** lacking the carbamoyl group in unit F of the moenomycin part[31] has been used to demonstrate complex formation between moenomycin and moenomycin-binding aptamers.[11]

Conjugates 6c and 6d

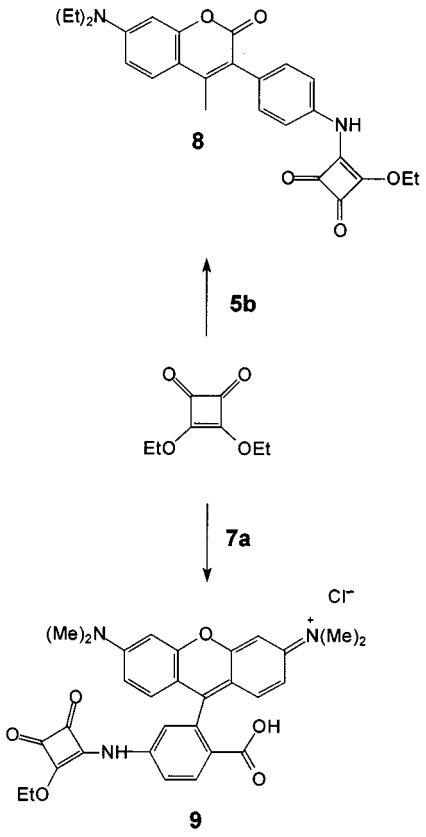
Fluorescein isothiocyanate (FITC, the 5-isomer) belongs to the most common fluorescent derivatization reagents. Fluorescein has an excitation maximum that closely matches the 488 nm spectral line of the argon ion laser, and at pH > 7 it has excellent fluorescence properties. However, it suffers from photobleaching, and the fluorescence is significantly reduced at pH < 7 (p*K*a = 6.4).[9] We coupled commercial FITC with **3b** under the conditions reported above. The ^1H NMR and the ESI FT ICR mass spectra of compound **6c** were in full accord with the proposed structure. The fluorescence spectra of **6c** were practically identical with the fluorescence spectra of fluorescein isothiocyanate measured in the same solvent system.[9]

4-Acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid disodium salt (SITS) was conjugated with **3b** because it is soluble in water. Conjugate **6d** was obtained in 63% yield. The structure of **6d** was elucidated from its ^1H and ^{13}C NMR spectra, with the aid of 2D NMR experiments. All carbon and proton signals were assigned for the fluorescent label (unit SITS), the nitroaromatic ring and the ethylenediamine-derived component (Ar and DAE), the triazole ring (TA) and the carboxypropionyl residue (A), the lipid component (I), and the glyceric acid moiety (H). The thioureido carbon signal at $\delta = 182.28$ and the signals of the *N*-acetyl, amido, and carbamoyl groups were also observed. ^1H and ^{13}C signals characteristic of the sugar part (signals of all anomeric hydrogen and carbon atoms, CH_2-6^D, CH_2-6^E, C-$2^{C,E}$) demonstrated that all sugar units were present. ESI FT ICR MS confirmed the structure **6d**. Comparison of the fluorescence spectra of **6d** with those of SITS (emission at 421 nm, excitation at 356 nm for SITS) in the same solvent system showed red shifts (+9 nm) in the emission and the excitation (+10 nm) maxima.

Control experiments were performed to test whether the isothiocyanates reacted selectively with the amino group of **3b**. Thus, moenomycin A (**1**) was treated with **5c** and the tetramethylrhodamine-derived isothiocyanate **7b** under exactly the same conditions as used for the preparation of **6a** and **6b**. After 72 h, no product formation could be observed by TLC.

Conjugation of Coumarin 5b and 6-Aminotetramethylrhodamine (7a) to 3b through the Squaric Acid Linker

Diethyl squarate as a bifunctional coupling reagent for bioconjugation was introduced by Tietze and coworkers.[20,21] The method is based on the fact that, under neutral conditions, only one of the ethoxy groups is replaced by primary or secondary amines whereas under basic conditions the corresponding symmetrical and unsymmetrical diamides, respectively, are formed. In the majority of cases, only aliphatic amines have been conjugated in this way. We found that diethyl squarate failed to react with **5b** and **7a** under the conditions used for the preparation of aliphatic amide esters (methanolic or ethanolic solution, room temperature, Scheme 4). However, **5b** did form amide



Scheme 4

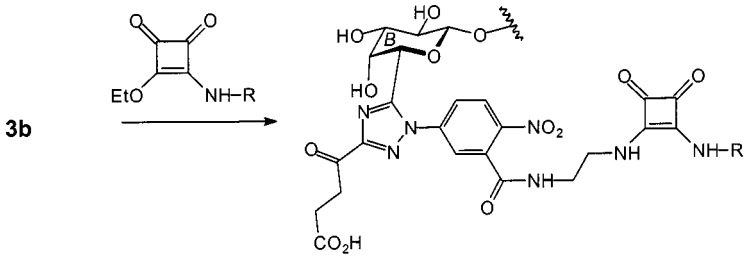
ester **8** in satisfactory yields when an excess of diethyl squarate was used and the reaction was performed in methanolic or ethanolic solution at room temperature in the presence of triethylamine. For the synthesis of **9** from diethyl squarate and **7a**, the reaction mixture was heated to 60 °C. In both cases the reaction stopped at the squaric amide ester stage. Previously, aromatic squaric amide esters had been briefly described by Maahs and Hegenberg,[32] but without experimental details.

The ^{1}H NMR and FAB mass spectra of both **8** and **9** were fully in agreement with the proposed structures. Treatment of **8** with **3b** was performed in methanol/Et_3N solution, and treatment of **9** in buffer solution (at pH = 9.0) because of the good solubility of **9** in water. In the case of **9**, an excess of **5** was used since only a small amount of the dye was available. Compounds **10a** and **10b** were carefully purified and were obtained in good yields. For compound **10a**, good quality ^{1}H and ^{13}C NMR spectra were obtained in CD_3OD/[D_6]DMSO solution. The signals in the ^{1}H and ^{13}C NMR spectra of **10a** were assigned by comparison with the ^{1}H and ^{13}C NMR spectra of **6a**. The ^{1}H NMR spectrum of **10b** in most solvents (CD_3OD and [D_6]DMSO or mixtures of them) displayed very broad signals. This may have been the result of micelle formation and/or the presence of the quinone form. However, characteristic signals were found in the ^{13}C NMR spectrum of **10b**, and the results of ESI FT ICR MS were also in full accord with the structure. The fluorescence spectra of compounds **10a** and **10b** were practically identical with those of compounds **6a** and **6b**, respectively (Table 2). For compounds containing the squaric acid linker, an additional maximum at ca. 351 nm (low intensity) in the excitation spectra was observed. From the observations reported here, it seems clear that, in cases in which an aromatic and an aliphatic amine are to be conjugated through diethyl squarate, the amide ester of the aromatic amine has to be formed first, and that this may then be treated with the aliphatic amine under basic conditions to provide the mixed diamide.

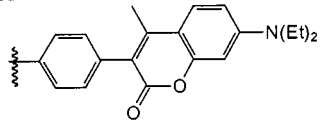
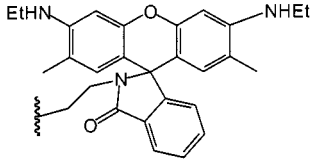
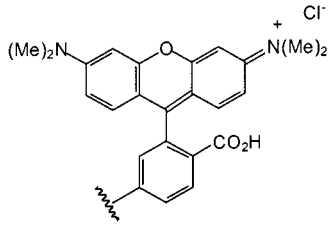
Synthesis of Rhodamine 6G Conjugate 10c

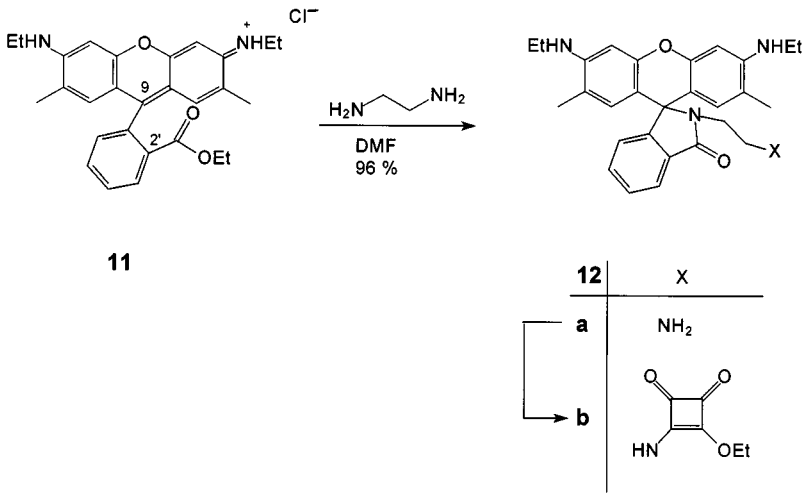
A major improvement in the synthesis of rhodamine conjugates was recently reported by Adamczyk and Grote.[33] These authors showed that rhodamine 2′-esters such as rhodamine 6G (**11**) react selectively with primary amines to provide rhodamine amides of type **12a**, which exist in their fluorescent open form at low to neutral pH and in the nonfluorescent spirolactam form (shown here) under basic conditions. The method of Adamczyk and Grote avoids the problem of working with mixture of 5- and 6-functionalized rhodamines as well as the need to synthesize the pure isomers (vide supra). Furthermore, the method does not necessitate working with fully *N*-alkylated rhodamines, and so

Table 2. Structures and fluorescence data of compounds **10a**−**10c**



10

10	R	Emission (excitation) λ_{max} [nm]	**10**	R	Emission (excitation) λ_{max} [nm]
a		475 (396)	**c**		557 (528, 351)
b		575 (546, 351)			



Scheme 5

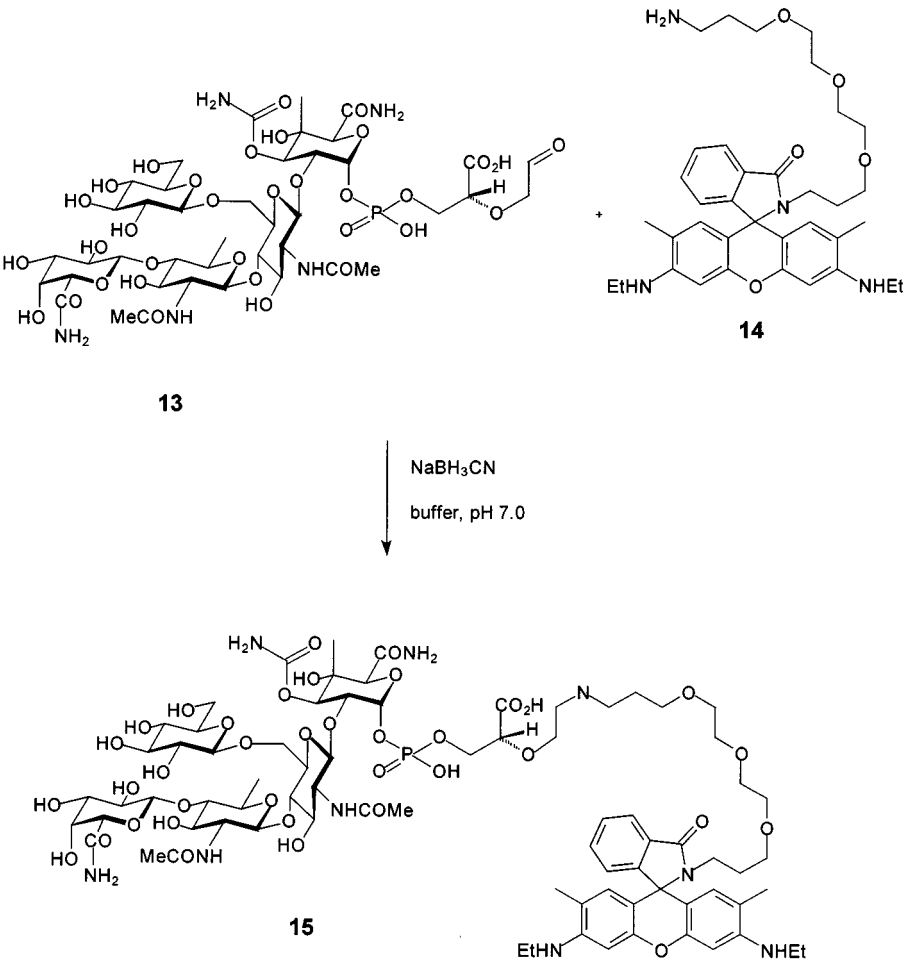
the inexpensive rhodamine 6G (**11**) can be used as the fluorophore. We combined the Adamczyk-Grote approach with the squaric acid linker technology. Thus, treatment of **11** with a large excess of ethylenediamine in DMF at room temp. afforded compound **12a** in over 90% yield (Scheme 5). All the spectra of **12a** (spiro carbon signal at $\delta = 65.11$) were in full agreement with the proposed structure. Replacement of the ethoxy group in diethyl squarate by the amino group of **12a** was performed in methanol/chloroform solution at room temp., and furnished amide ester **12b** in 79% yield. The proton and carbon signals of **12b** in the ^{1}H and ^{13}C NMR spectra were assigned with the aid of 2D NMR experiments. Hindered rotation around the C−NH bond in the squaric amide ester[20] resulted in the appearance of two signals for the CH_2-2^{AE} group at $\delta = 3.38$ and 3.44 and two NH (squaric acid part) signals at $\delta = 5.96$ and 6.93 (each corresponding to 0.5 H). The ethoxy group ^{1}H signals were also doubled. To define the CH_2-1^{AE} and CH_2-2^{AE} signals in the ^{1}H NMR spectrum (as well as in the ^{13}C NMR spectrum), HMBC was used. It was shown that the protons with signals at $\delta = 3.31$ had long-range couplings to the three carbon atoms C-2^{AE}, C-$9'^{R6G}$, C-3^{R6G}, and thus were the CH_2-1^{AE} protons. The other protons, with signals at $\delta = 3.38$, had long-range couplings to carbon atoms C-1^{AE} and C-3^{SA} and had to belong to the CH_2-2^{AE} group. In the ^{13}C NMR spectrum of **12b**, the spiro carbon signal (at $\delta = 65.71$) and the characteristic double signal of C-2^{AE} were observed. Compound **12b** was then conjugated with **3b** in the usual way to give **10c**. The structure of **10c** was elucidated from its high-quality ^{1}H and ^{13}C NMR spectra with the aid of 2D NMR experiments. All carbon and hydrogen signals of the rhodamine moiety (R6G), the squaric acid unit (SA), the nitroaromatic residue (Ar), the triazole ring (TA), the carboxypropionyl unit (A), and the lipid component (I) were assigned. The characteristic signals of the sugar carbon atoms (anomeric carbon atoms, C-$2^{C,E}$, C-$4^{C,E}$, C-$6^{D,E}$) and the signals of *N*-acetyl, carbamoyl, amido, and carboxy (H) groups were also identified. Distinction between the carbon signals of the AE and the DAE units was difficult because of the similar surroundings of these groups, and the assignments were based on comparison with the ^{13}C NMR spectrum of **12b**.

Synthesis of Rhodamine 6G Conjugate 15

We recently demonstrated that moenomycin derivatives lacking the lipid chain nevertheless bind to the enzyme PBP 1b through the sugar moiety.[16] Thus, fluorescent derivatives without the lipid chain may be of interest for study of the interaction between moenomycin derivatives and PBP 1b by fluorescence correlation spectroscopy (FCR). We prepared one compound of this type from aldehyde **13** by reductive amination. Compound **13** is available from moenomycin in high yield by ozonolysis. The amine component was prepared as follows. Rhodamine 6G was treated with 4,7,10-trioxatridecane-1,13-diamine as described above to furnish **14** in 73% yield. Reductive amination of **13** with **14** provided the desired conjugate **15**, albeit in low yield (18%, Scheme 6). The ^{1}H, ^{31}P, and ^{13}C NMR and the ESI mass spectra were fully in agreement with structure **15**.

Antibiotic Properties of Selected Moenomycin Conjugates

The MIC (Minimum Inhibitory Concentration) values against seven different *Staphylococcus aureus* strains (ATCC 25923, ATCC 29213, MRSA 1309, SG 511, PEG 18, PEG 5, KNS PEG 5) were determined by a serial double microdilution method on microtiter plates as described previously.[10] All compounds displayed antibiotic activity, although to a much smaller extent than moenomycin A (Table 3). This means that they should be useful in study by FCS of, for example, the interaction between moenomycin analogues and the transglycosylase in competition experiments.



Scheme 6

Table 3. Minimum inhibitory concentration of moenomycin-NH_2 and derivatives against a number of *Staph. aureus* strains

	MIC	
	μmol/L	μg/mL
1	0.0069	0.029
3b	0.15	0.27
6a	1.35	2.95
6b[a]	0.70	1.63
6c	0.58	1.25
6d	0.23	0.53
10c	1.62	3.75

[a] Result based on one measurement.

Conclusion

A number of fluorescent moenomycin derivatives have been prepared. Some of them have already shown their merits in biophysical studies of moenomycin membrane anchoring and intervesicle transfer, as well as in fluorescence correlation spectroscopy (FCS) investigation of the binding of moenomycin to aptamers.

Experimental Section

Gerneral: All O_2- or moisture-sensitive reactions were performed in oven-dried glassware under a positive pressure of argon. Liquids and solutions were transferred by syringe. Small-scale reactions were performed in Wheaton serum bottles sealed with aluminum caps with open top and Teflon-faced septum (Aldrich). "Usual workup" means partitioning of the reaction mixture between an aqueous phase and CH_2Cl_2, drying of the combined organic solutions with Na_2SO_4, and removal of solvent by distillation with a rotary evaporator (bath temperature 45 °C). Solvents were purified by standard techniques. The following materials and methods were used for chromatographic separations: flash chromatography (FC):[34] 32−63 μm silica gel (ICN Biomedicals); medium-pressure liquid chromatography (MPLC): 40-60 μm silica gel (Grace), Duramat pump (CfG); analytical TLC: Merck precoated 60 F_{254} silica gel plates (0.2 mm), spots were identified under a UV lamp (λ = 254 nm, Camag 29 200) and with a 2.22 mol/L H_2SO_4 solution containing $Ce(SO_4)_2{\cdot}4H_2O$ (10 g/L) and $H_3[PO_4(Mo_3O_9)_4]{\cdot}H_2O$ (25 g/L)[35] and heating at 140 °C, or an anisaldehyde reagent for carbohydrates [2 mL of anisaldehyde, 8 mL of conc. H_2SO_4 in ethanol (190 mL)]. Medium-pressure liquid chromatography (MPLC): LiChroprep RP-18 40−63 μm material (Merck) was used; the samples were applied to a precolumn (ca. 2 g of RP-18 material) and eluted at 1.5−2.5 bar with a Duramat (CFG) dosage pump. Analytical HPLC was performed with an HPLC system (Jasco) consisting of an intelligent PU-980 HPLC pump, a DG-980-05 3-

line degasser, an LG-980-02 ternary gradient unit, and an MD-910 multiwavelength detector. As eluent a mixture of buffer (prepared from 0.6 g of KH_2PO_4, 26.2 g of $K_2HPO_4 \times 3H_2O$, 3.0 g of 1-heptanesulfonic acid sodium salt monohydrate, and 1000 mL of water, pH = 8.0) and acetonitrile (60:40) was used. A 250 × 4.6 mm, nucleosil 300, C18, 5 μm column (Jasco) with a pre-column was used. Ultrafiltration (UF) was performed using gas-pressurized (N_2, 3.5 bar), stirred ultrafiltration cells (Amicon, model 8050, 50 mL cell capacity and model 8400, 400 mL capacity) with YM3 membrane type (Amicon, molecular weight cut-off 3000 Da). NMR and MS equipment: NMR: UNITY 400 (Varian), DRX 400 (Bruker), DRX 600 (Bruker), GEMINI 200 (Varian), GEMINI 2000 (Varian); MS: FAB MS: VG Autospec (Fisons, matrix: 3-nitrobenzyl alcohol), ESI MS: FT ICR MS APEX II (Bruker Daltonics, water-methanol). Following the molecular formula, two masses are always communicated: the first is calculated using the International Atomic Masses, the second is the monoisotopic mass. IR: Genesis FTIR (ATI Mattson). For the description of the NMR spectra the protons and carbon atoms are indexed according to the indices in the formulae. Fluorescence spectra were recorded with a Fluoromax-2 (SPEX) in MeOH/H_2O (1:1) solution at pH = 6.9. The fluorescence excitation and emission were corrected according to the lamp spectrum and according to the photomultiplyer sensitivity, respectively. The maxima of the excitation spectra indicate the UV/Vis absorption maxima. The MIC (minimum inhibitory concentration) values against seven different *Staphylococcus aureus* strains (ATCC 25923, ATCC 29213, MRSA 1309, SG 511, PEG 18, PEG 5, KNS PEG 5) were determined by a serial double microdilution method on microtiter plates (Iso-Sensitest medium, Oxoid). A series of decreasing concentrations of the compound under investigation was prepared in the medium. For inoculations, 1×10^5 cfu mL^{-1} were used. The MICs were determined (absence of visible turbidity) after 24 h at 37 °C. The MIC values were calculated as the average values from three measurements. For the NMR spectra of compounds **3c**, **3d**, **6a**, **6c**, **6d**, **10a**, **10b**, **10c**, and **15** see Supporting Information (see also the footnote on the first page of this article).

(*R*)-3-({(5*R*)-5-[3-(3-Carboxypropionyl)-1-(3-{[2-aminoethyl]-carbamoyl}-4-nitrophenyl)-1*H*-1,2,4-triazol-5-yl]-α-L-arabino-pyranosyl-(1→4)-2-acetamido-2,6-dideoxy-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-3-*O*-carbamoyl-4-*C*-methyl-α-D-glucopyranuronamidosyloxy}hydroxyphosphoryloxy)-2-[(2*Z*,6*E*,13*E*)-3,8,8,14,18-pentamethyl-11-methylene-2,6,13,17-nonadecatetraenyloxy]propionic Acid (3b): The preparation and spectroscopic data of this compound have been described elsewhere.[16] Here we want to emphasize that the NMR spectra were taken after RP18 chromatography [Kieselgel 60M C18, Macherey−Nagel, H_2O, 300 mL, then the compound was eluted with acetonitrile/water (1:2)]. The sample thus obtained contained small amounts of impurities originating from the column material. These impurities could be removed by FC [SiO_2, *n*PrOH/H_2O (7:3)] to give 269 mg (78%) of pure compound **3b**.

5-Amino-2-nitro-*N*-(2-piperazin-1-yl-ethyl)benzamide (Formula not Shown): Carbonyl diimidazole (267 mg, 1.6 mmol) was added to a solution of 5-amino-2-nitrobenzoic acid (200 mg, 1.1 mmol) in pyridine (20 mL), and the mixture was stirred at 20 °C for 30 min [TLC monitoring: methanol/$CHCl_3$ (1:1)]. A solution of 2-aminoethylpiperazine (350 mg, 2.7 mmol) in pyridine (20 mL) was then added, and the reaction mixture was stirred at 20 °C for 60 min and at 80 °C for 90 min. Solvent evaporation and FC [methanol/$CHCl_3$ (2:1)] provided 5-amino-2-nitro-*N*-(2-piperazin-1-yl-ethyl)-benzamide (270 mg, 98%). ^{13}C NMR (50 MHz, CD_3OD): δ = 36.9 (CH_2^{DAE}), 45.2 (CH_2^{PIP}), 53.9 (CH_2^{PIP}), 57.3 (CH_2^{DAE}), 112.4, 113.3 (C-6^{Ar}, C-4^{Ar}), 127.9 (C-3^{Ar}), 134.0 (C-1^{Ar}), 136.7 (C-2^{Ar}), 155.4 (C-5^{Ar}), 170.1 (amide CO).

(*R*)-3-({(5*R*)-5-[3-(3-Carboxypropionyl)-1-(3-{[2-piperazin-1-yl-ethyl]carbamoyl}-4-nitrophenyl)-1*H*-1,2,4-triazol-5-yl]-α-L-arabino-pyranosyl-(1→4)-2-acetamido-2,6-dideoxy-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-3-*O*-carbamoyl-4-*C*-methyl-α-D-glucopyranuronamidosyloxy}hydroxyphosphoryloxy)-2-[(2*Z*,6*E*,13*E*)-3,8,8,14,18-pentamethyl-11-methylene-2,6,13,17-nonadecatetraenyloxy)propionic Acid (3c): A solution of sodium nitrite (4.5 mg, 0.07 mmol) in water (1 mL) was added dropwise at 0 °C to a solution of 5-amino-2-nitro-*N*-(2-piperazin-1-yl-ethyl)-benzamide (20.0 mg, 0.07 mmol) in 9% HCl (0.5 mL). After 15 min at 0 °C, this solution was added dropwise to a solution of **1** (100 mg, 0.06 mmol) and sodium acetate (800 mg) in water (40 mL), and the mixture was stirred at 20 °C. Progress of the reaction was monitored by RP-HPLC-DAD [buffer/acetonitrile (59:41)]. After 48 h, the intermediate amidrazone (formula not shown) had been completely converted into triazole **3c**. Ultrafiltration followed by FC [$CHCl_3$/methanol/H_2O (18:11:2.7)] furnished **3c** (74.8 mg, 63%). R_t = 12.4 min, RP-HPLC [buffer/acetonitrile (59:41), λ_{max} = 279 nm]. $C_{82}H_{124}N_{11}O_{37}P$ (1886.91, 1885.79), ESI FT ICR MS: *m*/*z* = 941.8894 (calcd. 941.8876) $[M - 2\ H]^{2-}$, 952.8791 (calcd. 952.8786) $[M - 3\ H + Na]^{2-}$.

5-Amino-2-nitro-*N*-{2-[5-(2-oxohexahydrothieno[3,4-*d*]imidazol-6-yl)pentanoylamino]ethyl}benzamide (Formula not Shown): A solution of DCC (160 mg) in DMF (10 mL) was added dropwise to a solution of biotin (200 mg, 0.82 mmol) and *N*-hydroxysuccinimide (120 mg) in DMF (20 mL) and the mixture was stirred at 20 °C overnight. After filtration and solvent evaporation, the residue was redissolved in DMF (10 mL), a solution of **2** (320 mg of the diacetates, ca. 1 mmol) in DMF (5 mL) was added dropwise, and the mixture was stirred at 20 °C for 20 h. Solvent evaporation and FC [(i): $CHCl_3$/methanol (5:1); (ii) $CHCl_3$/methanol/ethyl acetate (5:10:5)] provided 250 mg (68%, based on biotin) of 5-amino-2-nitro-*N*-{2-[5-(2-oxohexahydrothieno[3,4-*d*]imidazol-6-yl)-pentanoylamino]ethyl}benzamide. ^{13}C NMR (50 MHz, APT, [D_6]DMSO, two sets of signals): δ = 25.5, 28.3, 28.5, 35.5, (C-2^{BTK}, C-3^{BTK}, C-1^{BTK},C-4^{BTK}), 38.3 (C-1^{DAE}, C-2^{DAE}), 55.7, 59.5, 59.6, 61.3, 61.4 (C-4^{BTR}, C-$3a^{BTR}$, C-$6a^{BTR}$), 112.2 (C-6^{Ar}), 112.7 (C-4^{Ar}), 127.7 (C-3^{Ar}), 133.2 (C-1^{Ar}), 137.1, 137.2 (C-2^{Ar}), 154.9, 154.9 (C-5^{Ar}), 163.2, 162.3 (CO^{BTR}), 167.6, 167.7 (*C*ONH^{Ar} signals), 172.8, 172.8 (*C*ONH^{BTK}). $C_{19}H_{26}N_6O_5S$ (450.51, 450.17), FAB MS: *m*/*z* = 451.2 $[M + H]^+$, 473.2 $[M + Na]^+$.

(*R*)-3-({(5*R*)-5-[3-(3-Carboxypropionyl)-1-(3-{[2-{[(3a*S*)-2-oxo-(3a*r*,6a*c*)-hexahydro-1*H*-thieno[3,4-*d*]imidazol-4*t*-yl]pentanoyl-amino}ethyl]carbamoyl}-4-nitrophenyl)-1*H*-1,2,4-triazol-5-yl]-α-L-arabinopyranosyl-(1→4)-2-acetamido-2,6-dideoxy-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxyβ-D-glucopyranosyl-(1→2)-3-*O*-carbamoyl-4-*C*-methyl-α-D-glucopyranuronamidosyloxy}hydroxyphosphoryloxy)-2-[(2*Z*,6*E*,13*E*)-3,8,8,14,18-pentamethyl-11-methylene-2,6,13,17-nonadecatetraenyloxy]propionic Acid (3d): A solution of sodium nitrite (9.9 mg, 0.07 mmol) in water (1 mL) was added dropwise at 0 °C to a solution of 5-amino-2-nitro-*N*-{2-[5-(2-oxohexahydrothieno[3,4-*d*]imidazol-6-yl)pentanoylamino]ethyl}benzamide (65 mg, 0.14 mmol) in 9% HCl (2 mL, dissolved at elevated temperature and then cooled to 0 °C). The mixture was left at 0 °C for 15 min and was then added dropwise to a solution of **1** (170 mg, 0.06 mmol) and sodium acetate (1000 mg) in water (30 mL). The

resulting mixture was stirred at 20 °C. Progress of the reaction was monitored by RP18 HPLC [buffer/acetonitrile (59:41), diode array detection]. After 48 h, the intermediate amidrazone (formula not shown) had been completely converted into **3d**. Ultrafiltration followed by FC [$CHCl_3$/methanol/H_2O (18:11:2.7)] furnished **3d** (157.1 mg, 72%) and, as a by-product, a 1-nitrosoimidazole derivative [RP HPLC, buffer/acetonitrile (63:37), R_t = 22 min]. ^{13}C NMR signal of the biotin part at δ = 156.8. $C_{88}H_{130}N_{13}O_{40}PS$ (2073.09, 2071.79), FAB MS: *m*/*z* = 2095.3 $[M + Na]^+$. Data of **3d:** RP18 HPLC [buffer/acetonitrile (63:37) R_t = 16 min, λ_{max} = 275 nm]. $C_{88}H_{131}N_{12}O_{39}PS$ (2044.09, 2042.81), FAB MS: *m*/*z* = 2067.0 $[M + Na]^+$, ESI FT ICR MS: *m*/*z* = 1020.3945 (calcd. 1020.3975) $[M - 2H]^{2-}$, 1031.3873 (calcd. 1031.3884) $[M - 3\ H + Na]^{2-}$, 679.9298 (calcd. 679.9292) $[M - 3\ H]^{3-}$.

3-(4-Acetamidophenyl)-7-(diethylamino)-4-methyl-2*H*-chromen-2-one (5a): A mixture of 7-diethylamino-4-methylcoumarin (0.5 g, 2 mmol), acetone (11 mL), sodium acetate (2 g), and $CuCl_2$ (80 mg) was stirred in a two-necked, round-bottomed flask equipped with a bubbler and a dropping funnel. A cold solution of the diazonium salt prepared from *N*-acetyl-*p*-phenylenediamine (0.45 g, 3 mmol) was added dropwise to this mixture at such a rate that the nitrogen evolution was slow (1−2 bubbles/s). When nitrogen formation was no longer observed, the mixture was acidified and extracted with chloroform. The aqueous layer was rendered alkaline and than extracted with diethyl ether. The ether and chloroform extracts were collected, washed with water, and dried with sodium sulfate. Solvent evaporation under reduced pressure and purification by FC [SiO_2, $CHCl_3$/toluene/methanol (10:10:1)] gave 207 mg (30%) of **5a**. R_f = 0.28 [$CHCl_3$/toluene/methanol (10:10:1)]. IR (KBr): $\tilde{\nu}$ = 3432, 1685, 1616, 1587, 1525, 1407, 1365, 1313, 1270 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$, HMQC, HMBC): δ = 1.23 [t, *J* = 7.0 Hz, 6 H, $N(CH_2CH_3)_2$], 2.16 (s, 3 H, CH_3CONH), 2.23 (s, 3 H, CH_3-9), 3.44 [q, *J* = 7.0 Hz, 4 H, $N(CH_2CH_3)_2$], 6.54 (d, J_{8-6} = 2.6 Hz, 1 H, 8-H), 6.64 (dd, J_{6-5} = 8.8 Hz, J_{6-8} = 2.6 Hz, 1 H, 6-H), 7.19 (d, $J_{2'-3'}$ = 8.8 Hz, 2 H, 2′-H, 6′-H), 7.46 (d, J_{5-6} = 8.8 Hz, 1 H, 5-H), 7.47 (d, $J_{3'-2'}$ = 8.4 Hz, 2 H, 3′-H, 5′-H), 7.81 (broad s, 1 H, CH_3CONH). ^{13}C NMR (50 MHz, $CDCl_3$, HMQC, HMBC): δ = 12.9 $[N(CH_2CH_3)_2]$, 16.8 (C-9), 24.9 (CH_3CONH), 45.2 $[N(CH_2CH_3)_2]$, 97.9 (C-8), 109.2 (C-6), 110.1 (C-4a), 120.3 (C-3′, C-5′), 121.4 (C-3), 126.6 (C-5), 131.3 (C-2′, C-6′), 131.4 (C-1′), 138.0 (C-4′), 149.3 (C-4), 150.8 (C-7), 155.5 (C-8a), 163.0 (C-2), 169.0 (CH_3CONH). $C_{22}H_{24}N_2O_3$ (364.44, 364.18), FAB MS: *m*/*z* = 365.2 $[M + H]^+$.

3-(4-Aminophenyl)-7-(diethylamino)-4-methyl-2*H*-chromen-2-one (5b): A solution of **5a** (10 mg, 0.027 mmol) in 6% HCl in ethanol was stirred at 80 °C. Progress of the reaction was monitored by TLC [$CHCl_3$/methanol (10:1)]. After 3 h, the mixture was neutralized with potassium carbonate and extracted with $CHCl_3$. The extract was washed with water and dried with Na_2SO_4. Solvents were evaporated under reduced pressure, and the residue was purified by FC [$CHCl_3$/methanol (10:1)] to give 8 mg (93%) of free amine **5b**. R_f = 0.57 [$CHCl_3$/methanol (10:1)]. IR (KBr): $\tilde{\nu}$ = 3428, 1695, 1616, 1583, 1521, 1467, 1407, 1349, 1301, 1270, 1174, 1143, 1074 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ = 1.22 [t, *J* = 7.0 Hz, 6 H, $N(CH_2CH_3)_2$], 2.26 (s, 3 H, CH_3-9), 3.43 [q, *J* = 7.0 Hz, 4 H, $N(CH_2CH_3)_2$], 6.55 (d, J_{8-6} = 2.6 Hz, 1 H, 8-H), 6.62 (dd, J_{6-8} = 2.6 Hz, J_{6-5} = 9.1 Hz, 1 H, 6-H), 6.74 (d, $J_{3'-2'}$ = 8.4 Hz, 2 H, 3′-H, 5′-H), 7.10 (d, $J_{2'-3'}$ = 8.4 Hz, 2 H, 2′-H, 6′-H), 7.44 (d, J_{5-6} = 9.1 Hz, 1 H, 5-H). ^{13}C NMR (50 MHz, $CDCl_3$): δ = 12.9 $[N(CH_2CH_3)_2]$, 16.8 (C-9), 45.2 $[N(CH_2CH_3)_2]$, 98.0 (C-8), 108.9 (C-6), 110.3 (C-4a), 115.3 (C-3′, C-5′), 125.7 (C-3), 126.5 (C-5), 131.9 (C-2′, C-6′), 146.3 (C-4′), 148.3 (C-4), 150.5 (C-7), 155.4 (C-8a), 162.9 (C-2). $C_{20}H_{22}N_2O_2$ (322.40, 322.17), FAB MS: *m*/*z* = 323.1 $[M + H]^+$.

7-(Diethylamino)-3-(4-isothiocyanatophenyl)-4-methyl-2*H*-chromen-2-one (5c): A solution of amine **5b** (112 mg, 0.347 mmol) in chloroform was slowly added to a well stirred heterogeneous mixture of $CHCl_3/H_2O$ (5:1) (6 mL), $CaCO_3$ (100 mg), and thiophosgene (45.1 mg, 0.392 mmol). The mixture was stirred at 20 °C and the reaction was monitored by TLC [$CHCl_3$/methanol (20:1)]. After 2 h, water was added and the mixture was extracted with $CHCl_3$. The organic extract was dried with sodium sulfate. Solvent was evaporated under reduced pressure, and the residue was purified by FC [$CHCl_3$/methanol (20:1)] to provide 68 mg (54%) of pure crystalline isothiocyanate **5c**. R_f = 0.86 [$CHCl_3$/methanol (20:1)]. M.p. 165 °C [$CHCl_3$/methanol (20:1)]. IR (KBr): $\tilde{\nu}$ = 3600−3400 (bs), 2113 (NCS), 1704, 1616, 1584, 1522, 1408, 1353, 1271, 1144, 1075 cm^{-1}. ^1H NMR (200 MHz, [D_6]DMSO): δ = 1.13 [t, *J* = 7.1 Hz, 6 H, $N(CH_2CH_3)_2$], 2.19 (s, 3 H, CH_3-9), 3.47 $[N(CH_2CH_3)_2$, hidden by the water signal], 6.56 (d, J_{8-6} = 2.6 Hz, 1 H, 8-H), 6.74 (dd, J_{6-8} = 2.6 Hz, J_{6-5} = 9.1 Hz, 1 H, 6-H), 7.37 (d, $J_{2'-3'}$ = 8.4 Hz, 2 H, 2′-H, 6′-H), 7.49 (d, $J_{3'-2'}$ = 8.4 Hz, 2 H, 3′-H, 5′-H), 7.59 (d, J_{5-6} = 9.1 Hz, 1 H, 5-H). ^{13}C NMR (50 MHz, $CDCl_3$): δ = 12.6 $[N(CH_2CH_3)_2]$, 16.4 (C-9), 45.2 $[N(CH_2CH_3)_2]$, 98.0 (C-8), 109.2 (C-6), 109.8 (C-4a), 119.9 (C-3), 125.7 (C-3′, C-5′), 126.3 (C-5), 130.7 (C-1′), 132.1 (C-2′, C-6′), 134.8, 136.0 (C-4′, NCS), 148.7 (C-4), 150.4 (C-7), 155.3 (C-8a), 161.7 (C-2). $C_{21}H_{20}N_2O_2S$ (364.46, 364.12), FAB MS: *m*/*z* = 365.1 $[M + H]^+$. Fluorescence spectrum [methanol/H_2O (1:1), 0.03 mg/5 mL]: excitation (478 nm): λ_{max} = 396 nm, emission (396 nm): λ_{max} = 478 nm.

6-Amino-3′,6′-bis(dimethylamino)spiro[1,3-dihydroisobenzofuran-1,9′-xanthen]-3-one (7a): This compound was prepared as described by Corrie and Craik.[30] ^1H NMR (200 MHz, CD_3Cl): δ = 2.97 [s, 12 H, $2N(CH_3)_2$], 4.11 (s, 2 H, 6-NH_2), 6.26 (d, J_{7-5} = 2.0 Hz, 1 H, 7-H), 6.41 (dd, $J_{2'-1'}$ = 8.7 Hz, $J_{2'-4'}$ = 2.6 Hz, 2 H, 2′-H, 7′-H), 6.46 (d, $J_{4'-2'}$ = 2.4 Hz, 2 H, 4′-H, 5′-H), 6.72 (d, $J_{1'-2'}$ = 8.6 Hz, 2 H, 1′-H, 8′-H), 6.74 (dd, J_{5-7} = 2.0 Hz, J_{5-4} = 8.2 Hz, 1 H, 5-H), 7.74 (d, J_{4-5} = 8.2 Hz, 1 H, 4-H). ^{13}C NMR (50 MHz, CD_3Cl, APT): δ = 40.9 $[N(CH_3)_2]$, 83.9 (C-9′), 99.2 (C-4′, C-5′), 106.2 (C-8′a, C-9′a), 108.2 (C-3a), 109.3 (C-2′, C-7′), 116.7 (C-5), 117.6 (C-7a), 120.5 (C-7), 126.9 (C-4), 129.5 (C-1′, C-8′), 152.6 (C-3′, C-6′), 153.2, 153.3 (C-4′a, C-10′a), 157.5 (C-6), 170.7 (C-3). $C_{24}H_{23}N_3O_3$ (401.46, 401.17), FAB MS: *m*/*z* = 424.2 $[M + Na]^+$, 402.1 $[M + H]^+$. Fluorescence spectrum [methanol/H_2O (1:1), 0.0024 mg/5 mL]: excitation (570 nm): λ_{max} = 547 nm, emission (547 nm): λ_{max} = 571 nm.

(*R*)-3-({(5*R*)-5-[3-(3-Carboxypropionyl)-1-(3-{[2-(3-{4-[7-(diethylamino)-4-methyl-2-oxo-2*H*-chromen-3-yl]phenyl}thioureido)ethyl]carbamoyl}-4-nitrophenyl)-1*H*-1,2,4-triazol-5-yl]-α-L-arabinopyranosyl-(1→4)-2-acetamido-2,6-dideoxy-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-3-*O*-carbamoyl-4-*C*-methyl-α-D-glucopyranuronamidosyloxy}hydroxyphosphoryloxy)-2-[(2*Z*,6*E*,13*E*)-3,8,8,14,18-pentamethyl-11-methylene-2,6,13,17-nonadecatetraenyloxy]propionic Acid (6a): The coumarin isothiocyanate **5c** (48 mg, 2 equiv.) in DMF (2 mL) was added to a solution of **3b** (120 mg, 0.066 mmol) in dry DMF (10 mL) and dry pyridine (3 mL). After 12 h, the mixture became clear and was stirred under argon and in darkness for an additional 12 h. Progress of the reaction was monitored by TLC [$CHCl_3$/methanol/H_2O (20:11:2)]. The solvents were then removed at ambient temperature under reduced pressure, and the residue was purified by FC [SiO_2; (i) $CHCl_3$/methanol (20:11), (2) $CHCl_3$/methanol/H_2O (20:11:2)] to give 73 mg

(51%) of pure product **6a**. For FRET experiments the substance was further purified by repeated MPLC [RP_{18}; acetonitrile/H_2O (1:1), R_f = 0.40]. $C_{99}H_{137}N_{12}O_{39}PS$ (2182.26, 2180.86), ESI FT ICR MS: *m*/*z* = 1089.4209 (calcd. 1089.4203) $[M - 2H]^{2-}$, 725.9463 (calcd. 725.9442) $[M - 3H]^{3-}$. Fluorescence spectrum [methanol/H_2O (1:1), 0.13 mg/5 mL]: excitation (476 nm): λ_{max} = 397 nm, emission (397 nm): λ_{max} = 476 nm.

Control Experiments − Treatment of 1 with 5c and 7b: Compounds **1** (3.2 mg, 0.002 mmol) and **5c** (0.73 mg, 0.002 mmol) in DMF (0.3 mL) and pyridine (0.025 mL) were stirred under argon at ambient temperature. The reaction was monitored by TLC [SiO_2; *n*PrOH/H_2O (7:3); RP_{18}; acetonitrile/H_2O (1:1)]. Even after 72 h, no products were observed. The same procedure was performed with **7b**. Again, no reaction product formation was observed. R_f = 0.09 [$CHCl_3$/methanol/H_2O (20:11:2)].

(*R*)-3-({(5*R*)-5-[1-(3-{[2-(3-{3-[3,6-Bis(dimethylamino)xanthylium-9-yl]-4-carboxyphenyl}thioureido)ethyl]carbamoyl}-4-nitrophenyl)-3-(3-carboxypropionyl)-1*H*-1,2,4-triazol-5-yl]-α-L-arabinopyranosyl-(1→4)-2-acetamido-2,6-dideoxy-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-3-*O*-carbamoyl-4-*C*-methyl-α-D-glucopyranuronamidosyloxy}hydroxyphosphoryloxy)-2-[(2*Z*,6*E*,13*E*)-3,8,8,14,18-pentamethyl-11-methylene-2,6,13,17-nonadecatetraenyloxy]-propionyl Chloride (6b): A solution of amine **7a** (19 mg, 0.047 mmol) was slowly added to a very well stirred suspension of thiophosgene (5.4 mg, 0.047 mmol) in water:chloroform, 3:1 (3 mL) and $CaCO_3$ (26 mg), and the mixture was stirred at 20 °C for 2 h. The reaction mixture was then neutralized with aq. KOH and extracted with $CHCl_3$. Solvent evaporation under reduced pressure and purification by FC [(i) $CHCl_3$, (ii) $CHCl_3$/methanol (20:1, 10:1, 5:1) gave 5.1 mg (21%) of the desired isothiocyanate **7b**. A solution of **7b** (5 mg, 0.01 mmol) in DMF (1 mL) and pyridine (0.2 mL) was added to a solution of **3b** (15 mg, 0.008 mmol) in DMF (1 mL). The resulting mixture was stirred in darkness at 20 °C under argon. Progress of the reaction was monitored by TLC [SiO_2; *n*PrOH/H_2O (7:2)] and [RP_{18}; $CHCl_3$/methanol (1:1)]. After 12 h, the reaction was complete (no **3b** was observed). Solvents were evaporated under reduced pressure and the crude product was purified by FC [SiO_2; *n*PrOH/H_2O (7:2)] and then several times by MPLC (RP_{18}) in different solvent systems [acetonitrile/H_2O (4:5, 2:3, 1:1)] to give 3.5 mg (18%) of pure **6b**. R_f = 0.13 [SiO_2; *n*PrOH/H_2O (7:2)], R_f = 0.42 [RP_{18}; $CHCl_3$/methanol (1:1)]. $C_{103}H_{138}N_{13}O_{40}PS$ (2261.32, 2259.86), FAB MS: *m*/*z* = 2260.6 $[M + H]^+$. ESI FT ICR MS: *m*/*z* = 2260.8743 (calcd. 2260.8700) $[M + H]^+$, 1149.9212 (calcd. 1149.9169) $[M + H + Na]^{2+}$, 1141.9105 (calcd. 1141.9338) $[M + H + K]^{2+}$, 1130.9037 (calcd. 1130.9350) $[M + 2H]^{2+}$, 766.9498 (calcd. 766.9472) $[M + 2H + K]^{3+}$, 754.2981 (calcd. 754.2892) $[M + 3H]^{3+}$. Fluorescence spectrum [methanol/H_2O (1:1), 0.10 mg/5 mL]: excitation (570 nm): λ_{max} = 544 nm, emission (544 nm): λ_{max} = 577 nm.

(*R*)-3-[((5*R*)-5-{3-(3-Carboxypropionyl)-1-[3-({2-[3-(3′,6′-dihydroxy-3-oxospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-5-yl)thioureido]-ethyl}carbamoyl)-4-nitrophenyl]-1*H*-1,2,4-triazol-5-yl}-α-L-arabinopyranosyl-(1→4)-2-acetamido-2,6-dideoxy-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-3-*O*-carbamoyl-4-*C*-methyl-α-D-glucopyranuronamidosyloxy)hydroxyphosphoryloxy]-2-[(2*Z*,6*E*,13*E*)-3,8,8,14,18-pentamethyl-11-methylene-2,6,13,17-nonadecatetraenyloxy)propionic Acid (6c): A solution of fluorescein isothiocyanate (isomer I, Fluka, 9 mg, 0.023 mmol) in dry DMF (2 mL) was added to a solution of **3b** (39 mg, 0.021 mmol) in dry DMF (2 mL) and dry pyridine (0.5 mL). The mixture was stirred in the dark at 20 °C under argon. Progress of the reaction was monitored by TLC [*n*PrOH/methanol/$CHCl_3$/H_2O (11:3:3:3)]. After 30 h, no **3b** was observed. Solvents were removed under reduced pressure, and the residue was purified by FC [*n*PrOH/methanol/$CHCl_3$/H_2O (11:3:3:3)]. Removal of organic solvents under reduced pressure and water by lyophilization gave 10 mg (26%) **6c**. R_f = 0.35 [*n*PrOH/methanol/$CHCl_3$/H_2O (11:3:3:3)]. $C_{99}H_{128}N_{11}O_{42}PS$ (2207.18, 2205.77), ESI FT ICR MS: *m*/*z* = 1120.8519 (calcd. 1120.8545) $[M + K - 3H]^{2-}$, 1112.8622 (calcd. 1112.8670) $[M + Na - 3H]^{2-}$, 1101.8754 (calcd. 1101.8759) $[M - 2H]^{2-}$, 746.9038 (calcd. 746.9006) $[M + K - H]^{3-}$, 741.5761 (calcd. 741.5753) $[M + Na - 4H]^{3-}$, 734.2470 (calcd. 734.2480) $[M - 3H]^{3-}$, 550.4358 (calcd. 550.4340) $[M - 4H]^{4-}$. Fluorescence spectrum [methanol/H_2O (1:1), 0.14 mg/5 mL]: excitation (515 nm): λ_{max} = 492 nm, emission (492 nm): λ_{max} = 521 nm.

(*R*)-3-[((5*R*)-5-{1-[3-({2-[3-[((*E*)-4′-Acetamido-2,2′-disulfostilben-4-yl)thioureido]ethyl}carbamoyl)-4-nitrophenyl]-3-(3-carboxypropionyl)-1*H*-1,2,4-triazol-5-yl}-α-L-arabinopyranosyl-(1→4)-2-acetamido-2,6-dideoxy-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-3-*O*-carbamoyl-4-*C*-methyl-α-D-glucopyranuronamidosyloxy)hydroxyphosphoryloxy]-2-[(2*Z*,6*E*,13*E*)-3,8,8,14,18-pentamethyl-11-methylene-2,6,13,17-nonadecatetraenyloxy]propionic Acid Disodium Salt (6d): A solution of 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid disodium salt (SITS) (11.9 mg, 0.023 mmol) in DMF (0.5 mL) and pyridine (0.1 mL) was added to a solution of **3b** (30 mg, 0.0165 mmol) in DMF (3 mL) and pyridine (0.45 mL). The mixture was stirred in darkness at 20 °C under argon for 9 h. Progress of the reaction was monitored by TLC [*n*PrOH/H_2O (7:3)]. The solvents were then removed under reduced pressure, and the residue was diluted with water (100 mL) and lyophilized. Water and Kieselguhr were added, and the water was lyophilized. The Kieselguhr containing the adsorbed compound was loaded onto the top of a FC column. Elution with *n*PrOH/H_2O (7:2) gave 25 mg (63%) of pure product **6d**. R_f = 0.53 [*n*PrOH/H_2O (7:3)]. $C_{95}H_{129}N_{12}Na_2O_{44}PS_3$ (2316.25, 2314.69), ESI FT ICR MS: *m*/*z* = 1156.3315 (calcd. 1156.3382) $[M - 2H]^{2-}$, 1145.3478 (calcd. 1145.3473) $[M - H - Na]^{2-}$, 770.5556 (calcd. 770.5562) $[M - 3H]^{3-}$, 763.2281 (calcd. 763.2289) $[M - 2H - Na]^{3-}$, 755.9020 (calcd. 755.9016) $[M - 2Na - H]^{3-}$, 572.1703 (calcd. 572.1697) $[M - 3H - Na]^{4-}$, 566.6751 (calcd. 566.6743) $[M - 2H - 2Na]^{4-}$. Fluorescence spectrum [methanol/H_2O (1:1), 0.21 mg/5 mL]: excitation (436 nm): λ_{max} = 346 nm, emission (346 nm): λ_{max} = 412 nm.

3-{4-[7-(Diethylamino)-4-methyl-2-oxo-2*H*-chromen-3-yl]phenyl}-4-ethoxy-3-cyclobutene-1,2-dione (8): A solution of **5b** (5 mg, 0.015 mmol) and 3,4-diethoxy-3-cyclobutene-1,2-dione (2.5 mg, 0.013 mmol) in ethanol (0.65 mL) was stirred at room temperature. Progress of the reaction was monitored by TLC [$CHCl_3$/ethyl acetate (4:1)]. Even after 5 h, however, no product spot was observed. A few drops of Et_3N and more diethyl squarate (10.2 mg, 0.06 mmol) were then added, and the resulting mixture was stirred at room temperature. After 4 h, no amine **5b** was observed by TLC [$CHCl_3$/ethyl acetate (4:1)]. Solvent was removed under reduced pressure and the residue was purified by FC [$CHCl_3$/ethyl acetate (4:1)] to give 6 mg (90%) of a specimen of **8** that was not completely free of solvent according to the ^1H NMR. R_f = 0.17 [$CHCl_3$/ethyl acetate (4:1)]. ^1H NMR (200 MHz, $CDCl_3$): δ = 1.22 [t, *J* = 7.0 Hz, 6 H, $N(CH_2CH_3)_2$], 1.49 (t, *J* = 7.0 Hz, 3 H, OCH_2CH_3), 2.25 (s, 3 H, CH_3-9^{Cou}), 3.43 [q, *J* = 7.0 Hz, 4 H, $N(CH_2CH_3)_2$], 4.88 (q, *J* = 7.0 Hz, 2 H, OCH_2CH_3), 6.55 (d, J_{8-6} = 2.6 Hz, 1 H, 8^{Cou}-H), 6.64 (dd, J_{6-8} = 2.6 Hz, J_{6-5} = 9.0 Hz, 1 H, 6^{Cou}-H),

7.27 (d, $J_{2'\text{-}3'}$ = 8.8 Hz, 2 H, 2′^{Cou}-H, 6′^{Cou}-H), 7.34 (d, $J_{3'\text{-}2'}$ = 8.6 Hz, 2 H, 3′^{Cou}-H, 5′^{Cou}-H), 7.46 (d, J_{5-6} = 9.0 Hz, 1 H, 5^{Cou}-H). $C_{26}H_{26}N_2O_5$ (446.50, 446.18), FAB MS: *m*/*z* = 460.0 [M + Na]$^+$, 447.5 [M + H]$^+$.

(*R*)-3-({(5*R*)-5-[3-(3-Carboxypropionyl)-1-(3-{[2-(2-{4-[7-(diethylamino)-4-methyl-2-oxo-2*H*-chromen-3-yl]anilino}-3,4-dioxo-1-cyclobuten-1-yl)ethyl]carbamoyl}-4-nitrophenyl)-1*H*-1,2,4-triazol-5-yl]-α-L-arabinopyranosyl-(1→4)-2-acetamido-2,6-dideoxy-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-3-*O*-carbamoyl-4-*C*-methyl-α-D-glucopyranuronamidosyloxy}hydroxyphosphoryloxy)-2-[(2*Z*,6*E*,13*E*)-3,8,8,14,18-pentamethyl-11-methylene-2,6,13,17-nonadecatetraenyloxy]propionic Acid (10a): A solution of **8** (5 mg, 0.011 mmol) in methanol (0.5 mL) and Et_3N (10 drops) was added to an emulsion of **3b** (20 mg, 0.011 mmol) in methanol (2 mL). The whole mixture became homogeneous and was stirred at ambient temperature under argon for 24 h. Progress of the reaction was monitored by TLC [$CHCl_3$/methanol/H_2O (20:12:3)]. The solvents were then evaporated under reduced pressure, and the residue was purified by FC [SiO_2; (i) $CHCl_3$/methanol (20:12), (ii) $CHCl_3$/methanol/H_2O (20:12:2)]. After solvent evaporation and lyophilization, 13 mg (53%) of pure **10a** was obtained. R_f = 0.25 [$CHCl_3$/methanol/H_2O (20:12:3)]. $C_{102}H_{137}N_{12}O_{41}P$ (2218.23, 2216.87), ESI FT ICR MS: *m*/*z* = 1118.4265 (calcd. 1118.4202) [M − 3 H + Na]$^{2-}$, 1107.4332 (calcd. 1107.4292) [M − 2 H]$^{2-}$, 737.9519 (calcd. 737.9502) [M − 3 H]$^{3-}$. Fluorescence spectrum [methanol/H_2O (1:1), 0.15 mg/5 mL]: excitation (475 nm): λ_{max} = 396 nm, emission (396 nm): λ_{max} = 475 nm.

9-[2-Carboxy-5-(2-ethoxy-3,4-dioxo-1-cyclobuten-1-ylamino)-phenyl]-3,6-bis(dimethylamino)xanthylium Chloride (9): Compound **7a** (10 mg, 0.025 mmol) and 3,4-diethoxy-3-cyclobutene-1,2-dione (19 mg, 0.11 mmol) in ethanol (1.5 mL) were stirred at 20 °C for 24 h {progress of the reaction was monitored by TLC [ethyl acetate/ethanol (5:1)]}. No product formation was observed. Triethylamine (1.1 mL) was then added, and the reaction mixture was heated to 60 °C and stirred at this temperature. The formation of a new compound was observed, and after 14 h, the mixture was allowed to cool to ambient temperature and Kieselguhr was added. Solvents were evaporated under reduced pressure and the residue was transferred onto the top of a FC column. Elution with ethyl acetate/ethanol (5:1), gave 9.7 mg (74%) of a product **9**, which was not completely pure according to the ^{1}H NMR. It contained some ethanol and a trace of triethylamine. R_f = 0.12 [ethyl acetate/ethanol (5:1)]. ^{1}H NMR (200 MHz, [D$_6$]DMSO): δ = 1.05 (t, *J* = 7.0 Hz, 3 H, OCH_2CH_3), 2.98 (s, 12 H, 2 $N(CH_3)_2^{TMR}$), 4.52 (q, *J* = 7.1 Hz, 2 H, OCH_2CH_3), 6.61 (broad signal, 6 H, 4′^{TMR}-H, 5′^{TMR}-H, 2′^{TMR}-H, 7′^{TMR}-H, 1′^{TMR}-H, 8′^{TMR}-H), 7.14 (broad signal, 1 H, 6^{TMR}-H), 7.55 (dd, J_{4-6} = 1.5 Hz, J_{4-3} = 8.4 Hz, 1 H, 4-H), 7.95 (d, J_{3-4} = 8.4 Hz, 1 H, 3-H), 11.06 (s, 1 H, COOH). $C_{30}H_{27}N_3O_6$ (525.56, 525.19), ESI FT ICR MS: *m*/*z* = 548.1785 (calcd. 548.1799) [M + Na]$^+$, 526.1969 (calcd. 526.1979) [M + H]$^+$.

(*R*)-3-[((5*R*)-5-{1-[3-({2-[(2-{[3′,6′-Bis(dimethylamino)-3-oxospiro-[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-6-yl]amino}-3,4-dioxo-1-cyclobuten-1-yl)amino]ethyl}carbamoyl)-4-nitrophenyl]-3-(3-carboxypropionyl)-1*H*-1,2,4-triazol-5-yl}-α-L-arabinopyranosyl-(1→4)-2-acetamido-2,6-dideoxy-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-3-*O*-carbamoyl-4-*C*-methyl-α-D-glucopyranuronamidosyloxy)hydroxyphosphoryloxy]-2-[(2*Z*,6*E*,13*E*)-3,8,8,14,18-pentamethyl-11-methylene-2,6,13,17-nonadecatetraenyloxy]propionic Acid (10b): Compounds **3b** (70 mg, 0.038 mmol) and **9** (9.7 mg, 0.018 mmol) in borax buffer (5 mL, pH = 9.03) were stirred under argon at 20 °C. The progress of the reaction was monitored by TLC [*n*PrOH/H_2O (7:2.5)]. When **9** could no longer be detected (after 72 h), *n*PrOH (14 mL) was added to the reaction mixture and this solution was transferred onto the top of a FC column. Elution with *n*PrOH/H_2O (7:2.5), solvent evaporation, and lyophilization gave 28 mg of a crude product. Further purification by FC [ethyl acetate/ethanol/H_2O (5:4:2.5)] provided 24 mg (58%) of pure product **10b**. R_f = 0.20 [*n*PrOH/H_2O (7:2.5)]. ^{1}H NMR {600 MHz, [D$_6$]DMSO and 400 MHz, CD_3OD/[D$_6$]DMSO (2:1)} displayed very broad signals, especially for the sugar and the aromatic protons. $C_{106}H_{138}N_{13}O_{42}P$ (2297.29, 2295.88), ESI FT ICR MS: *m*/*z* = 1157.9266 (calcd. 1157.9231) [M − 3 H + Na]$^{2-}$, 1146.9332 (calcd. 1146.9321) [M − 2 H]$^{2-}$, 764.2851 (calcd. 764.2854) [M − 3 H]$^{3-}$. Fluorescence spectrum [methanol/H_2O (1:1), 0.14 mg/5 mL]: excitation (573 nm): λ_{max} = 546 nm, λ_{max} = 354 nm, emission (546 nm): λ_{max} = 575 nm.

2-[3′,6′-Bis(ethylamino)-2′,7′-dimethyl-3-oxospiro[1*H*-isoindole-1,9′-[9*H*]xanthen]-2(3*H*)-yl]ethylamine (12a): A solution of commercial rhodamine 6G (**11**, 0.3 g, 0.62 mmol) in DMF (40 mL) was slowly added to a solution of ethylenediamine (0.6 mL, 14 equiv.) in DMF (20 mL), and the resulting mixture was stirred at 20 °C for 22 h. The solvent was then distilled off under reduced pressure (10^{-2} mbar), and the residue was purified by FC [methanol/ethanol/$CHCl_3$ (1:1:10)] to give 0.27 g (96%) of product **12a**, containing traces of ethanol and DMF according to ^{1}H NMR. R_f = 0.38 [methanol/ethanol/$CHCl_3$ (1:1:10)]. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.26 [t, *J* = 7.0 Hz, 6 H, 2 $NH(CH_2CH_3)$], 1.84 (s, 6 H, CH_3-11′, CH_3-12′), 2.19 (s, 2 H, NH_2), 2.34 (t, J_{18-17} = 6.0 Hz, 2 H, CH_2-2^{AE}), 3.14 [m, 6 H, CH_2-1^{AE}, 2 $NH(CH_2CH_3)$], 3.57 [broad t, 2 H, 2 $NH(CH_2CH_3)$], 6.18 (s, 2 H, 1′-H, 8′-H), 6.29 (s, 2 H, 4′-H, 5′-H), 7.00 (m, 1 H, 7-H), 7.39 (m, 2 H, 5-H, 6-H), 7.85 (m, 1 H, 4-H). ^{13}C NMR (50 MHz, $CDCl_3$, APT): δ = 14.6 [$NH(CH_2CH_3)$], 16.6 (C-11′, C-12′), 38.2 [$NH(CH_2CH_3)$], 40.5, 43.3 (C-1^{AE}, C-2^{AE}), 65.1 (C-9′), 96.4 (C-4′, C-5′), 105.8 (C-8′a, C-9′a), 117.9 (C-2′, C-7′), 122.7 (C-4), 123.7 (C-7), 128.0 (C-5), 128.1 (C-1′, C-8′), 130.9 (C-3a), 132.4 (C-6), 147.4 (C-3′, C-6′), 151.6 (C-4′a, C-10′a), 153.4 (C-1), 168.6 (C-3). $C_{28}H_{32}N_4O_2$ (456.58, 456.25), ESI FT ICR MS: *m*/*z* = 457.2593 (calcd. 457.2525) [M + H]$^+$.

3-({2-[3′,6′-Bis(ethylamino)-2′,7′-dimethyl-3-oxospiro[1*H*-isoindole-1,9′-[9*H*]xanthen]-2(3*H*)-yl]ethyl}amino)-4-ethoxy-3-cyclobutene-1,2-dione (12b): A solution of 3,4-diethoxy-3-cyclobutene-1,2-dione (0.27 g, 1.17 mmol) in methanol (2 mL) was added dropwise to a solution of **12a** (0.24 g, 0.53 mmol) in methanol (4 mL) and chloroform (3.5 mL), and the resulting mixture was stirred at 20 °C for 18 h. Progress of the reaction was monitored by TLC [$CHCl_3$/ethanol (20:1)]. Solvents were evaporated under reduced pressure and the residue was purified by FC [$CHCl_3$/ethanol (25:1)]. After removal of the solvents, the crude product was further purified by FC [(i) $CHCl_3$ (1500 mL), (ii) $CHCl_3$/ethanol (25:1)] to give 0.24 g (79%) of pure product **12b**. R_f = 0.31 [$CHCl_3$/ethanol (20:1)]. IR (KBr): $\tilde{\nu}$ = 3419, 1683, 1608, 1517, 1467, 1444, 1421, 1383, 1348, 1268, 1209 cm^{-1}. UV (CH_3OH): λ_{max} (ε) = 236.0 (66967), 251.5 (S) (49117), 300.5 (S) nm (13589). ^{1}H NMR (400 MHz, $CDCl_3$, ^{1}H,^{1}H COSY, HMQC, HMBC): δ = 1.33 [t, *J* = 7.2 Hz, 6 H, $NH(CH_2CH_3)$], 1.37/1.44 (two t, *J* = 7.4 Hz, 3 H, OCH_2CH_3), 1.89 (s, 6 H, CH_3-11′^{R6G}, CH_3-12′^{R6G}), 3.21 [q, *J* = 7.2 Hz, 4 H, $NH(CH_2CH_3)$], 3.31 (broad signal, 2 H, CH_2-1^{AE}), 3.38/3.44 (two broad signals, 2 H, CH_2-2^{AE}), 4.63/4.70 (two broad q, *J* = 7.4 Hz, 2 H, OCH_2CH_3), 5.96 (broad s, 0.5 H, NH-3^{SA}), 6.18 (s, 2 H, 1′^{R6G}-H, 8′ ^{R6G}-H), 6.34 (s, 2 H, 4′^{R6G}-H, 5′^{R6G}-H), 6.93 (broad signal, 0.5 H, NH-3^{SA}), 7.06 (m, 1 H, 7^{R6G}-H), 7.48 (m, 2 H, 5^{R6G}-

H, 6^{R6G}-H), 7.93 (m, 1 H, 4^{R6G}-H). ^{13}C NMR (50 MHz, $CDCl_3$, APT): δ = 14.9 [NH(CH_2CH_3)], 15.9 (OCH_2CH_3), 16.9 (C-11′^{R6G}, C-12′^{R6G}), 38.5 [NH(CH_2CH_3)], 40.9 (C-1^{AE}), 43.2, 45.2 (C-2^{AE}), 65.7 (C-9′^{R6G}), 69.5 (OCH_2CH_3), 96.6 (C-4′^{R6G}, C-5′^{R6G}), 104.9, 105.4 (C-8′a^{R6G}, C-9′a^{R6G}), 118.6 (C-2′^{R6G}, C-7′^{R6G}), 123.0 (C-4^{R6G}), 124.2 (C-7^{R6G}), 127.9 (?), 128.4 (C-5^{R6G}), 128.5 (C-1′^{R6G}, C-8′^{R6G}), 130.9 (C-3a^{R6G}), 133.1 (C-6^{R6G}), 147.9 (C-3′^{R6G}, C-6′^{R6G}), 151.9 (C-4′a^{R6G}, C-10′a^{R6G}), 153.7 (C-7a^{R6G}), 170.2 (C-3^{R6G}), 172.7 (C-3^{SA}), 176.9 (C-4^{SA}), 183.5, 191.0 (C-1^{SA}, C-2^{SA}). $C_{34}H_{36}N_4O_5$ (580.68, 580.27), ESI FT ICR MS: *m*/*z* = 581.2743 (calcd. 581.2765) $[M + H]^+$. Fluorescence spectrum [methanol/H_2O (1:1) (4 mL) + 0.2% TFA (1 mL), 0.045 mg/5 mL]: excitation (556 nm): λ_{max} = 529 nm, λ_{max} = 351 nm, emission (528 nm): λ_{max} = 556 nm.

(*R*)-3-{[(5*R*)-5-(1-{3-[(2-{[2-({2-[3′,6′-Bis(ethylamino)-2′,7′-dimethyl-3-oxospiro[1*H*-isoindole-1,9′-[9*H*]xanthen]-2(3*H*)-yl]ethyl}amino)-3,4-dioxo-1-cyclobuten-1-yl]amino}ethyl)-carbamoyl]-4-nitrophenyl}-3-(3-carboxypropionyl)-1*H*-1,2,4-triazol-5-yl)-α-L-arabinopyranosyl-(1→4)-2-acetamido-2,6-dideoxy-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-3-*O*-carbamoyl-4-*C*-methyl-α-D-glucopyranuronamidosyloxy]hydroxyphosphoryloxy}-2-[(2*Z*,6*E*,13*E*)-3,8,8,14,18-pentamethyl-11-methylene-2,6,13,17-nonadecatetraenyloxy)propionic Acid (10c): A suspension of **12b** (19 mg, 0.032 mmol) in methanol (1 mL) was added to a solution of **3b** (50 mg, 0.027 mmol) in methanol (10 mL) and triethylamine (0.2 mL). The whole mixture became homogeneous and was stirred at 20 °C under argon for 6 h. Progress of the reaction was monitored by TLC [*n*PrOH/H_2O (7:2)]. No product formation was observed. More triethylamine (0.5 mL) was added, and stirring was continued for an additional 44 h (until compound **3b** could no longer be observed by TLC). After removal of the solvents under reduced pressure, the crude product was purified by FC [*n*PrOH/H_2O (7:1.5)]. *n*-Propanol was distilled off under reduced pressure and water was removed by lyophilization to give 35 mg (54%) of pure product **10c**. R_f = 0.53 [*n*PrOH/H_2O (7:2)]. $C_{110}H_{147}N_{14}O_{41}P$ (2352.42, 2350.96), ESI FT ICR MS: *m*/*z* = 1185.4699 (calcd. 1185.4624) $[M + Na - 3H]^{2-}$, 1174.4780 (calcd. 1147.4714) $[M - 2H]^{2-}$, 782.6458 (calcd. 782.6450) $[M - 3H]^{3-}$. Fluorescence spectrum [methanol/H_2O (1:1) (4 mL) + 0.2% TFA (1 mL), 0.08 mg/5 mL]: excitation (550 nm): λ_{max}, 528 nm, λ_{max} = 351 nm, emission (528 nm): λ_{max} = 557 nm.

2-(3-{2-[2-(3-Aminopropoxy)ethoxy]ethoxy}propyl)-3′,6′-bis(ethylamino)-2′,7′-dimethylspiro[1*H*-isoindole-1,9′-[9*H*]xanthen]-3(2*H*)-one (14): 4,7,10-Trioxatridecane-1,13-diamine (589.7 mg, 2.67 mmol) was added slowly to a solution of rhodamine 6G (**11**, 427 mg, 0.89 mmol) in DMF (50 mL). The mixture was stirred at 60 °C for 24 h. Solvent evaporation and FC [$CHCl_3$/methanol/NH_3 (5:1:0.05)] followed by solvent evaporation and lyophilization provided pure **14** (402 mg, 73%). ^1H NMR (400 MHz, D_2O, ^1H, ^1H COSY): δ = 1.25 (t, CH_3-9^C, CH_3-9^D), 1.69−1.75 (m, CH_2-2^A), 1.84 (s, CH_3-7^C, CH_3-7^D), 1.82−1.90 (m, CH_2-9^A), 3.04 (dd, $J_{9A\text{-}10A}$ = 6.1 Hz, CH_2-10^A), 3.14−3.20 (m, CH_2-8^C, CH_2-8^D), 3.26 (m, CH_2-1^A), 3.48 (dd, $J_{2A\text{-}3A}$ = 6.0 Hz, CH_2-3^A), 3.50−3.57 (m, CH_2-4^A, CH_2-5^A, CH_2-6^A, CH_2-7^A), 3.58 −3.62 (m, CH_2-8^A), 6.06 (s, H-5^C, H-5^D), 6.30 (s, H-2^C, H-2^D), 6.97−6.99 (m, H-2^B), 7.47−7.51 (m, H-3^B, H-4^B), 7.81−7.83 (m, H-5^B). ^{13}C NMR (50 MHz, D_2O): δ = 14.8 (CH_3-9^C, CH_3-9^D), 17.2 (CH_3-7^C, CH_3-7^D), 27.9 (C-9^A), 29.1 (?), 30.2 (C-2^A), 36.4 (C-8^C, C-8^D), 38.5 (?), 39.1 (C-1^A), 40.2 (C-10^A), 67.2 (C-8^A), 69.7, 70.3, 70.5, 70.8, 70.9, 71.1, 71.4 (C-3^A − C-7^A), 97.4 (C-5^C, C-5^D), 106.1 (C-1^C, C-1^D), 119.8 (C-3^C, C-3^D), 123.4 (C-2^B), 125.0 (C-5^B), 129.0 (C-2^C, C-2^D), 129.4 (C-4^B), 132.1 (C-6^B), 133.9 (C-3^B), 149.3 (C-4^C, C-4^D), 153.2 (C-6^C, C-6^D), 155.1 (C-1^B), 163.7 (?) 169.8 (C-7^B). $C_{36}H_{48}N_4O_5$ (616.80, 616.36), FAB MS: *m*/*z* = 617.4 $[M + H]^+$.

(*R*)-2-{16-[3′,6′-Bis(ethylamino)-2′,7′-dimethyl-3-oxospiro[1*H*-isoindole-1,9′-[9*H*]xanthen]-2(3*H*)-yl]-7,10,13-trioxa-3-azahexadecyloxy}-3-({β-D-galactopyranuronamidosyl-(1→4)-2-acetamido-2,6-dideoxy-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→6)]-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-3-*O*-carbamoyl-4-*C*-methyl-α-D-glucopyranuronamidosyloxy}hydroxy-phosphoryloxy)propionic Acid (15): Compounds **13** (80 mg, 67 μmol) and **14** (154.3 mg, 268 μmol), on reductive amination as described in ref.[36] provided − after purification [(i) LH-20, water/methanol (1:4); (ii) Dowex 50Wx8; (iii) FC, ethyl acetate/2-propanol/water (6:4:2); (iv) LH-20, water/methanol (1:4)] and lyophilization − 21.3 mg (18%) of pure **15**. $C_{77}H_{114}N_9O_{37}P$ (1788.76, 1787.70), ESI FT ICR MS: *m*/*z* = 892.8440 (892.8454) $[M - 2H]^{2-}$.

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