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Heterobifunctional Trityl Derivatives as Linking Reagents for the Recovery of Nucleic Acids after Labeling and Immobilization

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Abstract: The synthesis of two heterobifunctional 4,4'-dimethoxytrityl derivatives carrying a N-hydroxysuccinic acid group as active ester in *para* **5a** or *meta* position **5b** is described. While **5a** has been synthesized previously via six steps a novel and more efficient four step synthesis for both compounds is introduced here. The *meta* compound **5b** provides for a more acid labile trityl ether linkage compared to the *para* derivative **5a**. Using these compounds as linking agents biopolymers in particular nucleic acids can easily be recovered after labeling or immobilization by treatment with a mild acid such as 80 % aqueous acetic acid.

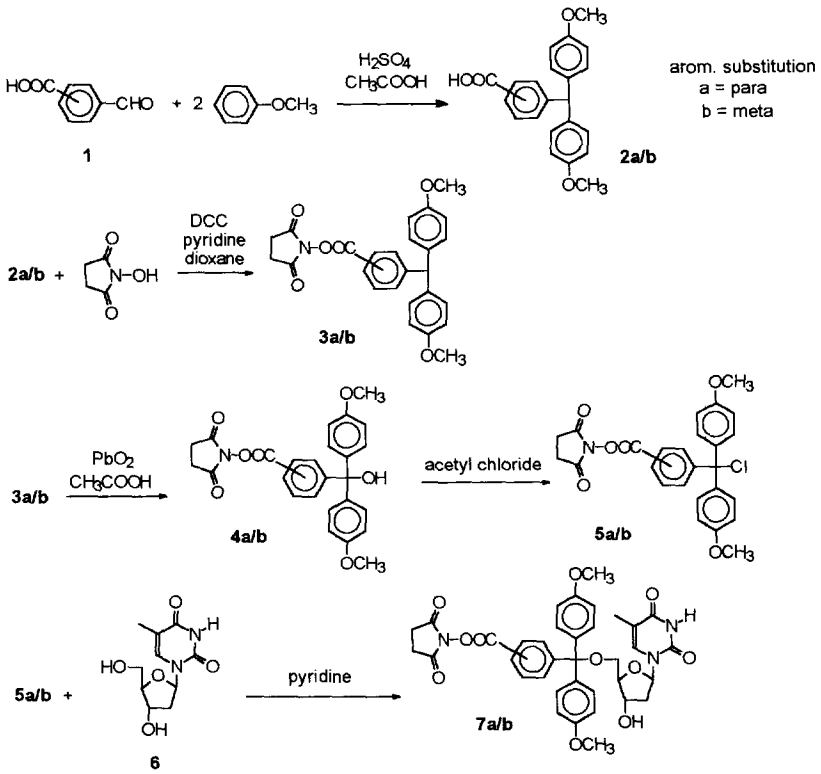
INTRODUCTION

There are numerous applications for labeling oligonucleotides and nucleic acids. Labeling is especially useful for the detection of the amplification products of polymerase chain reaction (PCR)[1,2] and necessary for DNA sequencing,[3,4] immobilization is an essential part of solid phase biotechnology[5] and solid phase DNA sequencing.[6] Most techniques for covalent labeling and immobilization are hampered by the fact that the biomolecule cannot be regenerated in its original form because the linking chemistry does not allow a selective cleavage. The selective reduction of a disulfide linkage has been proposed.[7] This approach, however, suffers from the difficulties involved in handling thiolated material and in removing the excess of reducing reagent such as dithiothreitol. The high demand for methods which offer a cleavable bond between the label or the polymeric support and the nucleic acid is demonstrated by the widespread use of the biotin-streptavidin system,[8] e.g. for DNA sequencing.[6,9] This approach, however, has two drawbacks. At first, although of non-covalent nature, the bond is essentially irreversible; very harsh conditions are necessary to overcome the strong interactions. In order to avoid this the biotin-streptavidin system has been combined with a linking disulfide group.[7] Secondly, the size of the streptavidin molecule limits the amount of biomolecules bound to a polymer surface for immobilization. Use of a bifunctional 4,4'-dimethoxytrityl group forming an acid labile trityl ether bond and having an active ester functionality for the attachment of labels or polymer supports does not have the above mentioned disadvantages. In contrast it reacts regioselectively with primary OH groups, can be cleaved with a mild acid such as aqueous acetic acid, which can easily be removed by evaporation, lyophilization or extraction and should allow for a very dense functionalization of polymeric surfaces. Previously we described the synthesis of N-succinimidyl-4-[bis-(4-methoxyphenyl)]-chloromethyl-benzoate, **5a**, in a demanding six-step synthesis.[10] We also observed that due to the -I- and -M-effect of the ester functionality in *para* position the trityl ether bond demonstrated a significant increase in stability over the conventionally used 4,4'-dimethoxytrityl ether

bonds. In this paper we therefore describe a rapid synthetic route of only four simple steps using cheap starting materials for the synthesis of the *para* and *meta* active esters of 4,4'-dimethoxytrityl chloride, the reaction with the 5'-OH group of deoxynucleosides, the immobilization to membranes and the stability of the trityl ether bond towards acidic cleavage.

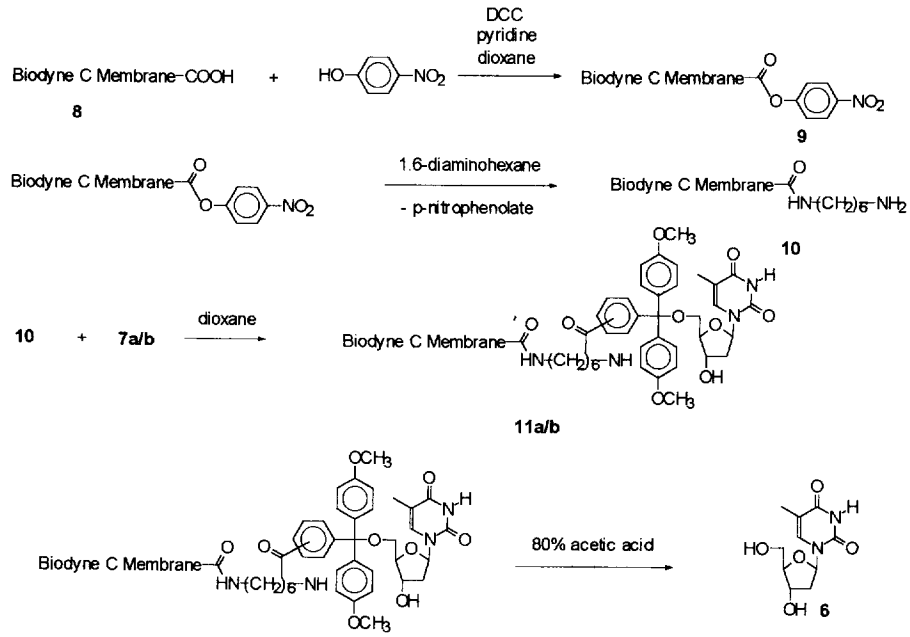
RESULTS AND DISCUSSION

In the previously published procedure[10] the starting material for 5a is 4-bromobenzoyl chloride and the key step a Grignard reaction after protection of the acid chloride functionality via 2-oxazoline ring formation. The procedure presented here for the synthesis of the *para* (**5a**) and *meta* trityl chloride derivative **5b** can be described as being very simple and versatile regarding the design of differently functionalized trityl chloride derivatives. The *meta* trityl chloride derivative **5b** has not been described before. The synthetic steps for both **5a** and **5b** are summarized in scheme 1. Starting material is 4- or 3-carboxybenzaldehyde **1a/b** which directly reacts with 4-methoxybenzene in a twofold aromatic electrophilic substitution in presence of concentrated sulfuric acid to form the appropriate triphenylmethane derivatives **2a/b** as crystalline compounds. Purification of the raw reaction mixture can be performed either by silica gel column chromatography or crystallization followed by Soxhlet extraction to remove polar side products. The corresponding active esters **3a/b** are obtained in high yields from **2a/b** through a condensation reaction with N-hydroxysuccinimide (NHS) mediated by N,N'-diyclohexyl-carbodiimide (DCC). The addition of water at the end of the reaction can be omitted. Purification by crystallization from ethanol would be only necessary if side products are present as detected by thin layer chromatography. **3a/b** are transformed to the dimethoxytriphenylmethylcarbinol (DMT) derivatives **4a/b** *via* oxydation with freshly prepared lead dioxide.[11] The raw material is purified by silica gel column chromatography in the presence of small amounts of pyridine to prevent trityl ether formation with ethanol which is being used as co-eluent. **4a/b** are obtained as crystalline compounds from acetone/hexane and treated with acetyl chloride according to published procedures[10] to furnish the title compound **5a** as colorless crystalline solid in excellent yield and purity. Compound **5b** could be obtained in good quality as checked by thin layer chromatography; it could not be crystallized and was transformed into a light pink solid by lyophilization from dioxane. Compounds were characterized by ^{1}H and ^{13}C NMR, mass spectrometry and elementary analyses. Regioselective tritylation of a deoxynucleoside such as deoxythymidine **6** at the 5'-hydroxyl group is performed as described previously[12].



Scheme 1

The NHS-DMT derivatives of deoxythymidine **7a/b** serve here as model compounds to investigate coupling to and cleavage off a membrane support. These reactions are shown in scheme 2. The Biodyne C membrane **8** bearing carboxyl groups is transformed into the active ester **9** in a reaction with 4-nitrophenol and N,N'-dicyclohexylcarbodiimide (DCC) in dioxane. The activated membrane **9** is treated with a dioxane solution of 1,6-diaminohexane which serves as a spacer for the nucleoside **6** or oligonucleotide[13] to be anchored to the surface and as a nucleophile for the reaction with the NHS-DMT nucleoside **7a/b**. The reaction with 1,6-diaminohexane is followed by the spectrophotometric determination of the 4-nitrophenolate anion at 411 nm released upon reaction with the nucleophile. According to our experience it is of importance for solid phase reactions to integrate into the reaction scheme steps which allow for a quantitation of functional groups on the surface and to monitor reactions on the polymer support. Quantitation revealed a loading of 130 to 138 pmol aminohexyl groups per mm^2 membrane. The NHS-DMT nucleosides **7a/b** were dissolved in a mixture of dioxane, N,N-dimethylformamide, pyridine, triethylamine and coupled *via* the active ester functionality to the primary amino group on the surface of the Biodyne membrane.



Scheme 2

The concept to recover the unmodified nucleoside after cleavage of the trityl ether bond was tested by treatment of the membrane **11a/b** with 80% aqueous acetic acid at room temperature. At different time intervals aliquots were analyzed by high performance liquid chromatography (HPLC) using isocratic conditions and detection with a photodiodearray detector to determine the rate of cleavage and the purity and integrity of the released deoxynucleoside. Results of the cleavage reaction are shown for the *para* and *meta* compound **11a/b** (figure 1a). Purity and integrity of the recovered deoxythymidine is shown in figure 1b; these results are identical for the *para* derivative **11a** (not shown). In case of the *para* compound **11a** quantitative cleavage needed eight hours; this trityl ether bond is therefore considerably more stable than the 4,4'-dimethoxytrityl ether bond which according to current DNA synthesis protocols[14] can be removed in two hours. As expected cleavage of the *meta*-NHS-DMT trityl ether bond in **11b** can be accomplished more rapidly compared to the *para*-NHS-DMT derivative in only three hours and hence is almost as reactive as the standard DMT group. Half live times of the cleavage reaction are 105.0 and 38.8 minutes for the *para*- and *meta*-NHS-DMT tritylether bond respectively. The HPLC data reveal that coupling and cleavage can be performed in a selective way and that the immobilized deoxynucleoside can be recovered in unmodified form and without impurities. Experiments are under way in which this procedure is adopted for the immobilization of oligonucleotides of defined sequence.[13]

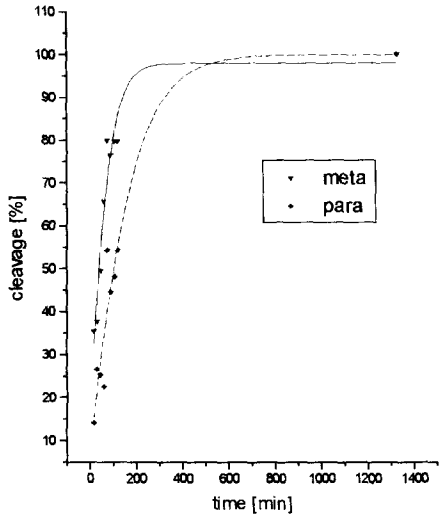


Figure 1a: Rate of cleavage of deoxythymidine from membranes **11a/b** with aqueous 80% acetic acid at room temperature as analyzed by HPLC (figure 1b).

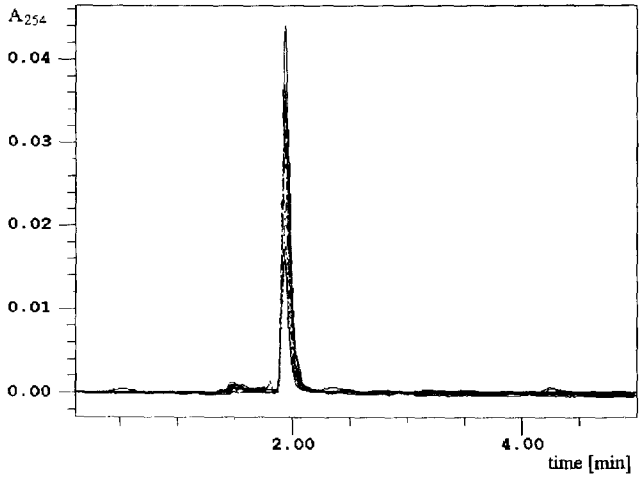


Figure 1b : The diagram represents the superposition of nine individual HPLC injections corresponding to the nine different time intervalls of the cleavage reaction from **11b**.

EXPERIMENTAL SECTION

^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Bruker AMX400 and a AC250-P instrument. Samples were dissolved in deuterochloroform with tetramethylsilane as internal standard, unless otherwise stated. Chemical shifts are given in ppm. Mass spectra were obtained on a Finnigan MAT 311 A mass spectrometer under EI conditions. Melting points are uncorrected. Elementary analyses were performed by the analytical department of the Institute of Organic Chemistry, University of Hamburg. Thin layer chromatography was carried out on 60 PF_{254} silica gel coated alumina sheets (Merck, Darmstadt; No.5562). Column chromatography was performed using silica gel from Merck. HPLC results were obtained on a Waters chromatography systems 625 LC with a photodiodearray detector 996 and using reversed phase columns (39 x 300 mm, Nova Pak C 18, 60 Å, 4 µm particles, software: Millenium 2.0, isocratic solvent system with acetonitrile/water: 65/35, v/v, flow rate: 1 ml/min, temperature : 30 °C). Spectrophotometric measurements in the UV/VIS region were performed on a Beckman UV35 spectrophotometer. Solvents were dried and purified before use according to standard procedures. Extractions were monitored by thin layer chromatography to optimize completion of extraction.

4-[Bis-(4-methoxyphenyl)]-methyl-benzoic acid (2a)

4-Carboxybenzaldehyde **1a** (46.9 g, 312 mmol) and 4-methoxybenzene (78.4 g, 725 mmol) were stirred in 1 l glacial acetic acid to dissolve most of the material. The mixture was cooled in an ice bath and immediately concentrated sulfuric acid (281.0 g) added dropwise. The reaction mixture was then stirred for about 24 h at room temperature until thin layer chromatography (dichloromethane/methanol: 8/2, v/v) demonstrated quantitative conversion. The reaction mixture was poured into 4 l ice/water and the aqueous phase extracted several times with ethyl acetate. The combined organic phase was successively washed with water and saturated sodium chloride solution and dried over Na_2SO_4. Solvents were evaporated and the remaining acetic acid removed by threefold coevaporation with toluene. The orange and oily raw reaction mixture was refluxed with petroleum ether, the petroleum ether phase decanted and cooled for crystallization (yield:10.0 g, 9%). The residue solidified at 4 °C and was recrystallized from ether (22.0 g, 20%). The mother liquor was evaporated and the solid residue Soxhlet extracted with petroleum ether (30-50 °C). Total yield: 57.8 g (53%) .

^1H NMR ($CDCl_3$): δ 3.78 (s, 6H, -OCH_3), 5.5 (s, 1H, R_3C-H), 6.83 (d, 4H, CH_3O-aryl-H , ortho), 7.0 (d, 4H, CH_3O-aryl-H , meta) , 7.22 (d, 2H, HOOC-aryl-H , meta), 8.03 (d, 2H, HOOC-aryl-H, ortho). - *^{13}C NMR* ($CDCl_3$, internal standard $CDCl_3$ at 77.00 ppm) δ 55.27 and 55.23 (q,-OCH_3 and d, R_3C-H, position not defined), 113.84 (d, C-H, CH_3O-aryl , ortho), 129.45 (d, C-H, HOOC-aryl , meta), 130.24 (d, C-H, HOOC-aryl , ortho and d, CH_3O-aryl, meta) ,127.24, 135.44, 151.08 (s, CR_3,aryl), 158.21 (s, R_2C-OCH_3, aryl), 171.94 (s, -COOH). - *Elementary Analysis* (%): Found: C, 75.46/75.79; H, 5.56/5.64; $C_{22}H_{20}O_4$ requires C, 75.85; H, 5.79. - *MS*: m/z (rel. intensity): m/z calculated for C_{22} H_{20} O_4 (M^+): 348; found: 348 (83), 227 (100). - *m.p.*: 144-46 °C.

3-[Bis-(4-methoxyphenyl)]-methyl-benzoic acid (2b)

3-Carboxybenzaldehyde (obtained according to lit.[15]) was treated as described for the synthesis of **2a**. Purification of the raw product can be accomplished either by crystallization from ethanol and subsequent Soxhlet extraction (see above) or by silica gel column chromatography (Merck, Darmstadt; No.9385; using a step gradient from dichloromethane to dichloromethane/methanol: 99/1,v/v) and subsequent crystallization from ethanol; **2b** is obtained with yields above 50%.

^1H NMR: ($CDCl_3$): δ 3.77 (s, 6H, -OCH_3), 5.5 (s, 1H, R_3C-H), 6.83 (d, 4H, CH_3O-aryl-H, ortho), 7.0 (d, 4H, CH_3O-aryl-H, meta), 7.36 (m, 2H, R_2N-OOC-aryl-H, meta and para), 7.88 (s, 1H, R_2N-OOC-aryl-H, isolated), 7.95 (d, 1H, R_2N-OOC-aryl-H, ortho). - *^{13}C-NMR*: ($CDCl_3$): δ 55.02 (d, R_3C-H), 55.24 (q, -OCH_3), 113.85 (d, C-H, CH_3O-aryl, ortho), 128.16 (d, C-H, HOOC-aryl, ortho), 128.48 (d, C-H, HOOC-aryl, meta or para), 130.23 (d, C-H, CH_3O-aryl, meta), 130.94 (d, C-H, HOOC-aryl, ortho, isolated), 134.73 (d, C-H, HOOC-aryl, meta or para), 129.36, 135.70, 145.29 (s, CR_3 , aryl), 158.17 (s, R_2C-OCH_3, aryl), 172.07 (s, -COOH). - *Elementary Analysis* (%): Found: C, 75.95/75.61; H, 5.77/5.79; $C_{22}H_{20}O_4$ requires C,

75.85; H, 5.79. *MS*: m/z (rel. intensity): m/z calculated for $C_{22}H_{20}O_4$ (M^+):348; found:348 (75), 227 (100). - *m.p.*: 144 °C

N-Succinimidyl-4-[bis-(4-methoxyphenyl)]-methyl-benzoate (3a)

Compound **2a** (6.59 g, 18.9 mmol) and N-hydroxysuccinimide (2.21 g, 19.2 mmol) were dissolved in dry dioxane (90 ml) and dry pyridine (4.9 ml). After addition of N,N'-dicyclohexylcarbodiimide (DCC) (4.50 g, 21.8 mmol) the mixture was stirred at room temperature until thin layer chromatography (dichloromethane/methanol: 96/4, v/v) revealed quantitative conversion (4-18 h). Stirring was continued for 2 h after addition of 1.6 ml water, the N,N'-dicyclohexylurea removed by filtration, the precipitate washed with dioxane until no UV absorbing material could be detected. An excess of water was added to the dioxane solution and the mixture extracted with dichloromethane. The organic phase was washed once with water and dried with Na_2SO_4. The solvent was evaporated, the residue azeotropically dried with toluene, dissolved in a small amount of dichloromethane and remaining dicyclohexylurea removed by filtration. Dichloromethane was evaporated and the product crystallized at 4 °C. To remove remaining DCC the crystalline product is triturated with hexane. **3a** was obtained as a colorless solid; yield: 7.46 g (89%).

^1H NMR : ($CDCl_3$): δ 2.9 (s, 4H, -CO-C$\underline{H}_2$-C$\underline{H}_2$-CO-), 3.78 (s, 6H, -OC$\underline{H}_3$), 5.52 (s, 1H, R_3C-$\underline{H}$), 6.84 (d, 4H, CH_3O-aryl-$\underline{H}$, ortho), 6.98 (d, 4H, CH_3O-aryl-$\underline{H}$, meta), 7.25 (d, 2H, R_2N-OOC-aryl-$\underline{H}$,meta), 8.05 (d, 2H,R_2N-OOC-aryl-$\underline{H}$,ortho). - *^{13}C NMR*: ($CDCl_3$): δ 25.69 (t, -CO-$\underline{C}H_2$-$\underline{C}H_2$-CO-), 55.27 and 55.3 (q, -O$\underline{C}H_3$ and d, $R_3\underline{C}$-H), 113.93 (d, $\underline{C}$-H, CH_3O-$\underline{aryl}$, ortho), 129.85 (d, $\underline{C}$-H, R_2N-OOC-$\underline{aryl}$, meta), 130.26 (d, $\underline{C}$-H, CH_3O-$\underline{aryl}$, meta), 130.65 (d, $\underline{C}$-H, R_2N-OOC-$\underline{aryl}$, ortho), 123.0, 135.1, 152.56 (s, $\underline{C}R_3$, aryl), 158.33 (s, $R_2\underline{C}$-OCH$_3$, aryl), 161.75 (s, -$\underline{C}$O-NR$_2$), 169.23 (s, -$\underline{C}$OOR). - *Elementary Analysis* (%): Found: C, 69.82/69.86;H, 4.99/4.92;N, 3.14/3.14; $C_{26}H_{23}NO_6$ requires C, 70.1; H, 5.2; N, 3.14. - *MS*: m/z (rel. intensity): m/z calculated for $C_{26}H_{23}NO_6$ (M^+): 445; found: 445 (27), 331 (100). - *m.p.*: 183-86 °C

N-Succinimidyl-3-[bis-(4-methoxyphenyl)]-methyl-benzoate (3b)

3b was prepared according to the procedure given above for **3a** with the exception that no water was added after the reaction and that extraction with water was omitted. N,N'-dicyclohexylurea was removed by filtration and the product purified by crystallization from ethanol. Yield: 88%.

^1H NMR: ($CDCl_3$): δ 2.85 (s, 4H, -CO-C$\underline{H}_2$-C$\underline{H}_2$-CO-), 3.78 (s, 6H, -OC$\underline{H}_3$), 5.50 (s, 1H, R_3C-$\underline{H}$), 6.83 (d, 4H, CH_3O-aryl-$\underline{H}$, ortho), 6.99 (d,4H, CH_3O-aryl-$\underline{H}$, meta), 7.40 (m, 2H, R_2N-OOC-aryl-$\underline{H}$, meta and para), 7.88 (s, 1H, R_2N-OOC-aryl-$\underline{H}$, ortho, isolated), 7.98 (d, 1H, R_2N-OOC-aryl-$\underline{H}$, ortho). - *^{13}C NMR*: ($CDCl_3$): δ 25.66 (t, -CO-$\underline{C}H_2$-$\underline{C}H_2$-CO-), 54.92 (d, $R_3\underline{C}$-H), 55.25 (q, -O$\underline{C}H_3$), 113.92 (d, $\underline{C}$-H, CH_3O-$\underline{aryl}$, ortho), 128.51 (d, $\underline{C}$-H, R_2N-OOC-$\underline{aryl}$, ortho), 128.84 (d, $\underline{C}$-H, R_2N-OOC-$\underline{aryl}$, meta or para), 130.24 (d, $\underline{C}$-H, CH_3O-$\underline{aryl}$, meta), 131.24 (d, $\underline{C}$-H, R_2N-OOC-$\underline{aryl}$, ortho, isolated), 135.9 (d, $\underline{C}$-H, R_2N-OOC-$\underline{aryl}$, meta or para), 125.16, 135.31, 145.86 (s, $\underline{C}R_3$, aryl), 158.25 (s, $R_2\underline{C}$-OCH$_3$, aryl), 161.92 (s, -$\underline{C}$O-NR$_2$), 169.24 (s, -$\underline{C}$OOR).- *Elementary Analysis* (%): Found: C ,69.66/ 69.82; H, 5.28/ 5.30; N, 3.19/ 3.17; $C_{26}H_{23}NO_6$ requires C, 70.1;H,5.2; N, 3.14. - *MS*: m/z (rel. intensity): m/z calculated for $C_{26}H_{23}NO_6$ (M^+): 445; found: 445 (36), 331 (100). - *m.p.*: 141 °C

N-Succinimidyl-4-[bis-(4-methoxyphenyl)]-hydroxymethyl-benzoate (4a)

Compound **3a** (4.00 g, 8.98 mmol) was dissolved in 130 ml glacial acetic acid and freshly prepared lead dioxide (2.99 g, 12.5 mmol) was added and the mixture placed into a preheated oil bath (100-130 °C) until a clear solution was obtained. Another 314 mg (1.31 mmol) lead dioxide were added and dissolved. The reaction was monitored by thin layer chromatography (dichloromethane/methanol: 99/1, v/v). During the reaction a side product appears which travels between the educt **3a** and product **4a**. The reaction was terminated when the UV intensity of the residual **3a** spot equals the side product. The reaction mixture was poured on ice/water (500 ml) and extracted with ethyl acetate . The organic phase was extracted with water (3 x 400 ml) and dried over Na_2SO_4. Solvents were evaporated and some remaining acetic acid removed by co-evaporation with toluene. The raw product (4.62 g) was dissolved in dichloromethane and purified by silica gel 60 H (Merck, Darmstadt; No.7736, 200 g) column chromatography; elution was carried out in presence of 0.03 % pyridine with dichloromethane/ethanol (99.6/0.4, v/v). Fractions containing **4a** were combined and the solvents evaporated. The

residue was dissolved in some acetone and crystallized after addition of an excess of hexane. Yield: 2.91 g (70%). 496 mg (about 10%) of the starting material were recovered (recrystallization from ethanol). *^1H NMR*: ($CDCl_3$): δ 2.78 (s, 1H, R_3C-O<u>H</u>), 2.88 (s, 4H, -CO-C<u>H</u>$_2$-C<u>H</u>$_2$-CO-), 3.80 (s, 6H,-OC<u>H</u>$_3$), 6.85 (d, 4H, CH_3O-aryl-<u>H</u> , ortho), 7.15 (d, 4H, CH_3O-aryl-<u>H</u>, meta), 7.5 (d, 2H, R_2N-OOC-aryl-<u>H</u>, meta), 8.07(d, 2H, R_2N-OOC-aryl-<u>H</u>, ortho). - *^{13}C NMR* : ($CDCl_3$): δ 25.69 (t, -CO-<u>C</u>H$_2$-<u>C</u>H$_2$-CO-), 55.32 (q, -O<u>C</u>H$_3$), 81.32 (s, R_3<u>C</u>-OH), 113.51 (d, <u>C</u>-H, CH_3O-<u>aryl</u>, ortho), 128.2 (d, <u>C</u>-H, R_2N-OOC-<u>aryl</u> , meta), 129.12 (d, <u>C</u>-H, CH_3O-<u>aryl</u>, meta), 130.2 (d, <u>C</u>-H, R_2N-OOC-<u>aryl</u>, ortho), 123.65, 138.43, 154.58 (s, <u>C</u>R$_3$, aryl), 159.01 (s, R_2<u>C</u>-OCH$_3$, aryl), 161.68 (s, -<u>C</u>O-NR$_2$), 169.21 (s, -<u>C</u>OOR).- *Elementary Analysis* (%): Found: C, 67.60/67.62; H, 4.81/4.82; N,3.02/3.01; $C_{26}H_{23}NO_7$ requires C, 67.67; H, 5.02; N, 3.04. - *MS*: m/z (rel. intensity): m/z calculated for $C_{26}H_{23}NO_7$ (M^+): 461; found: 461 (15), 444 (5, M-OH $^+$), 243 (100), 135 (90). - *m.p.*: 186-88 °C (Lit.[10]): 188-90 °C).

N-Succinimidyl-3-[bis-(4-methoxyphenyl)]-hydroxymethyl-benzoate (4b)

Synthesis of **4b** was carried out essentially as described for **4a**. The yield is similar to **4a**. *^1H NMR*: ($CDCl_3$): δ 2.04 (s, 1H, R_3C-O<u>H</u>), 2.85 (s, 4H, -CO-C<u>H</u>$_2$-C<u>H</u>$_2$-CO), 3.78 (s, 6H, -OC<u>H</u>$_3$), 6.84 (d, 4H, CH_3O-aryl-<u>H</u>, ortho), 7.14 (d, 4H, CH_3O-aryl-<u>H</u>, meta), 7.42 (m, 1H, R_2N-OOC-aryl-<u>H</u>, meta), 7.6 (d, 1H, R_2N-OOC-aryl-<u>H</u>, para), 8.03 (d, 1H,R_2N-OOC-aryl-<u>H</u>, ortho), 8.14 (s, 1H, R_2N-OOC-aryl-<u>H</u>, ortho, isolated). - *^{13}C NMR* ($CDCl_3$,internal standard $CDCl_3$ at 77.00 ppm): δ 25.63 (t, -CO-<u>C</u>H$_2$-<u>C</u>H$_2$-CO-), 55.25 (q, -O<u>C</u>H$_3$), 81.07 (s, R_3<u>C</u>-OH), 113.46 (d, <u>C</u>-H, CH_3O-<u>aryl</u>, ortho), 128.33 (d, <u>C</u>-H, R_2N-OOC-<u>aryl</u>, meta), 129.08 and 129.18 (d, <u>C</u>-H, CH_3O-<u>aryl</u>, meta and d, <u>C</u>-H, H_2N-OOC-<u>aryl</u>, ortho, position not defined), 129.45 (d, <u>C</u>-H, R_2N-OOC-<u>aryl</u>, ortho, isolated C-H), 134.45 (d, <u>C</u>-H, R_2N-OOC-<u>aryl</u>, para), 124.77, 138.62, 148.52 (s, <u>C</u>R$_3$,aryl), 158.89 (s, R_2<u>C</u>-OCH$_3$, aryl), 161.87 (s, -<u>C</u>O-NR$_2$), 169.21 (s, -<u>C</u>OOR). - *Elementary Analysis* (%): Found: C, 67.42/67.6; H, 4.83/4.87; N, 2.79/2.82; $C_{26}H_{23}NO_7$ requires C, 67.67; H, 5.02; N, 3.04. - *MS* : m/z (rel. intensity) : m/z calculated for $C_{26}H_{23}NO_7$ (M^+): 461; found : 461 (16), 444 (8, M-OH$^+$), 243 (100), 135 (72). - *m.p.*: 162° C

N-Succinimidyl-3/4-[bis-(4-methoxyphenyl)]-chloromethyl-benzoate (5a/b)

Compounds **4a** and **4b** respectively were treated under reflux with freshly distilled acetyl chloride as described previously.[10] **5a** was obtained in excellent yield as colorless crystalline solid from acetyl chloride/ether. **5b** was obtained as pink solid after evaporation of the acetyl chloride and lyophilization from dioxane.

Tritylation of deoxythymidine (6) with 5b to 7b

Compound **5b** (500 mg, 1.04 mmol) and deoxythymidine **6** (240 mg, 992 μmol) were dissolved in dry pyridine (6 ml) and stirred for 3 h at room temperature. The reaction mixture was poured on ice/water, three times extracted with chloroform and the combined organic phase dried over Na_2SO_4. The solvent was evaporated and **7b** purified by preparative thin layer chromatography (silica gel plates, 2 mm thick, Merck, Darmstadt; No.1.05717) with dichloromethane/methanol (9/1,v/v). **7b** was crystallized from toluene/cyclohexane. The corresponding compound **7a** is prepared in the same way. Yields and purities are similar to the published values for **7a**.[10]

7a :

^1H-NMR: ($CDCl_3$): δ 1.58 (s, 3H, C<u>H</u>$_3$ of thymine), 2.23/2.40 (m, 2H, H2'^{a}/H2'^{b}), 2.87 (s, 4H, -CO-C<u>H</u>$_2$-C<u>H</u>$_2$-CO-), 3.33/3.40 (m, 2H, H5'^{a}/H5'^{b}), 3.78 (s, 6H, -OC<u>H</u>$_3$), 4.06 (m, 1H, H4'), 4.52 (m, 1H, H3'), 6.35 (t, 1H, H1'), 6.85 (d, 4H, CH_3O-aryl-<u>H</u>, ortho), 7.25 (d, 4H, CH_3O-aryl-<u>H</u>, meta), 7.43 (s, 1H, H6), 7.6 (d, 2H, R_2N-OOC-aryl-<u>H</u>, meta), 8.06 (d, 2H, R_2N-OOC-aryl-<u>H</u>, ortho), 9.1 (s, 1H, N-<u>H</u> of thymine).- *m.p.*: 139-44 °C (decomp.)

7b :

^1H-NMR: ($CDCl_3$): δ 1.53 (s, 3H, C<u>H</u>$_3$ of thymine),2.32/2.40 (m, 2H, H2'^{a}/H2'^{b}), 2.87 (s, 4H, -CO-C<u>H</u>$_2$-C<u>H</u>$_2$-CO-), 3.32/3.45 (m, 2H, H5'^{a}/H5'^{b}), 3.8 (s, 6H, -OC<u>H</u>$_3$), 4.06 (m, 1H, H4'), 4.60 (m, 1H, H3'), 6.35 (t, 1H, H1'), 6.85 (d, 2H, CH_3O-aryl-<u>H</u>, ortho), 7.25 (d, 4H, CH_3O-aryl-<u>H</u>, meta), 7.42 (m, 1H, R_2N-OOC-

aryl-H, meta), 7.55 (s, 1H,H6), 7.64 (d, 1H, R_2N-OOC-aryl-H, para), 7.98 (d, 1H, R_2N-OOC-aryl-H, ortho), 8.34 (s, 1H, R_2N-OOC-aryl-H, ortho, isolated), 9.14 (s, 1H, N-H of thymine). - *m.p.*: 104-06 °C (decomp.)

Anchoring of deoxythymidine derivative 7a/7b to the Biodyne C membrane (9)

A piece of Biodyne C membrane (1 cm^2) was treated for 15 min with 1M hydrochloric acid, washed with water until hydrochloric acid was removed quantitatively, washed with ethanol and air-dried. The membrane was then suspended in a mixture of 4-nitrophenol (177 mg, 1.44 mmol), 4.6 ml dioxane and 0.32 ml dry pyridine and the condensation initiated by addition of a 1M solution of N,N'-dicyclohexylcarbodiimide (DCC) (0.1 ml) in dry dioxane. After gentle shaking for 2 h at room temperature the membrane was washed with ethanol (20 ml) until the filtrates demonstrated complete absence of 4-nitrophenol by treatment with concentrated ammonium hydroxide (absorbance measured at 411 nm). Subsequently the membrane was treated. with a 1M solution (0.5 ml) of 1,6-diaminohexane in dry dioxane. The solution turned immediately yellow. The membrane was washed with dioxane (5 ml) and the combined solution analyzed spectrophotometrically at 411 nm. With a calibration curve the loading was determined to be between 130 and 138 pmol per mm^2 membrane **10**. The membrane was washed successively with water until the filtrate was neutral (20 ml) and ethanol and air-dried. A 1 cm^2 piece of membrane **10** was now separately treated for 10 min at room temperature under gentle shaking with either **7a** or **7b** (4 mg, 5.8 µmol) in a mixture of 14.8 µl dioxane/pyridine (10/1, v/v) and 40.5 µl N,N-dimethylformamide/triethylamine (8/1, v/v). The membranes were washed with dichloromethane (about 20 ml each) until addition of 5% dichloroacetic acid in dichloromethane (v/v) to the filtrates revealed complete absence of trityl-containing material. Finally membranes **11a** and **11b** were air-dried and stored at -20 °C.

Rate of Cleavage of deoxythymidine (6) from the membranes 11a and 11b

Each membrane was treated with 80% aqueous acetic acid (200 µl) under gentle shaking at room temperature. Aliquots of 10 µl were withdrawn every 15 min, lyophilized, dissolved in 10 µl acetonitrile/water (65/35, v/v) and analyzed on a Waters HPLC system (Waters 625 LC system, Waters 996 PDA photodiodearray detector; column: NovaPak C18, 80 Å, 4 µm particles, 3.9 mm x 300 mm), isocratic solvent system with acetonitrile/water (65/35, v/v), flow rate :1ml min^{-1}, temperature 30°C. A final aliquot was taken after 24 h. Figure 1a represents the time course of the cleavage reaction and figure 1b shows the HPLC profiles of the analyzed aliquots withrawn at different time intervals.

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