

New Transmembrane Polyene Bolaamphiphiles as Fluorescent Probes in Lipid Bilayers**

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The former view of the cell lipid membrane as a somewhat random and disordered distribution of lipids and proteins has given way to one characterized by a complex lateral organization, with nm-sized domains in which specific lipids and proteins are assembled to carry out a particular process.^[1] The study of the morphology, composition, regulation, and persistence time of these structures is an experimental challenge that has been taken up by fluorescence probe methods. In the case of lipid–lipid and lipid–protein interactions, the dependence of the resonant transfer of electronic excitation on the proximity of donor and acceptor probe molecules can provide very useful information in the 10-nm range.^[2] We have previously developed a series of fluorescent fatty acids that contain five conjugated double bonds at the end of a flexible linear chain.^[3] These probes accurately report the changes in order and dynamics of the lipid environment in artificial and natural membranes. Furthermore, the large spectral overlap of the polyene absorption with the tryptophan fluorescence from membrane proteins and peptides is very convenient for energy-transfer experiments. However, the mobility of the energy-accepting polyene group in fluid bilayers may introduce some uncertainty into the distance evaluation. This would also be the case in other possible applications of the probes, such as the detection of oxidizing radicals by fluorescence quenching. To restrict the mobility of the chromophore, a linear structure with two polar terminal groups, that is, a bolaform amphiphile similar to the membrane lipids of *Archaeobacteria*^[4] would be ideal, with the additional advantage of precisely locating the sensing group of the probe in the bilayer. Bolaamphiphiles can be constructed with the appropriate length to reach both external membrane surfaces with the distal polar groups, and with a sensitive group in the lipophilic central part of the molecule.^[5] Some transmembrane bolaamphiphiles have been produced previously for altering specific membrane properties: for example, compounds with *p*-benzoquinone or anthraquinone groups to change the redox behavior,^[6] or conjugated polyenes derived from the natural product bixine to modify the charge-transfer rate across the bilayer.^[7]

Furthermore, bolaamphiphiles that contain photochemically reactive benzophenone^[8] or diazirine^[9] groups, or emitting fluorene^[10] or anthracene groups,^[11] have been also synthesized. A different but interesting fluorescent bolaform structure was also produced by linking two rhodamine 101 dye molecules with a C₃₂ linear chain.^[12]

Herein we describe the synthesis of symmetrical bolaamphiphiles with four, five, or six conjugated double bonds in the center of the molecule, with terminal methyl ester (**4**) or carboxylic acid (**5**) groups (Scheme 1), and with the appropriate length for spanning the lipid bilayer (Figure 1). The intermediates in the synthesis of the former compounds are conjugated polyenediynes diesters **3** that might also be useful transmembrane probes as such or in the form of free acids.

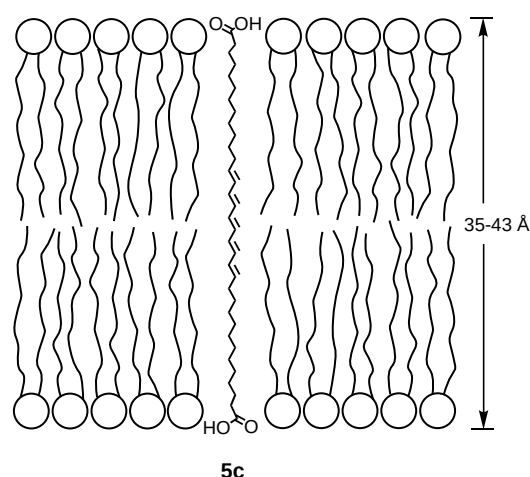
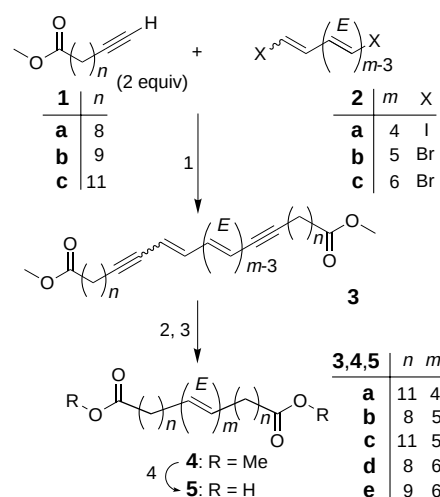


Figure 1. Schematic representation of the fluorescent polyene bolaamphiphile **5c** in a phospholipid bilayer.

Probes **5** were synthesized in four steps (Scheme 1), each with a yield of 95 % or higher: 1) Sonogashira–Hagihara double cross-coupling^[13] between the terminal acetylene



Scheme 1. 1) 1/2 3:1 (mol), [Pd(PPh₃)₂Cl₂] (10 mol %), CuI (20 mol %), Et₃NH, THF, Ar, room temperature, 3 h, >95 %; 2) Zn(Cu/Ag), MeOH/H₂O 1:1, room temperature, 24 h, ≥95 %; 3) I₂ (trace), hexane, Ar, reflux, 15 min, ≥95 %; 4) KOH (10 equiv), EtOH (95 %), Ar, reflux, 5 h; then 0 °C, HCl (5 %), ≥95 %.

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group of methyl esters **1** and conjugated 1, ω -dihalopolyenes **2** with two, three, or four double bonds to give the polyenediynes **3**; 2) partial reduction of the triple bonds of **3** with activated Zn^[14] to yield the corresponding polyene diesters with two more *Z* double bonds; 3) isomerization of the resulting polyenes with iodine to form the corresponding all-(*E*) isomers **4**; and 4) alkaline hydrolysis of **4** to yield the diacids **5**. As the cross-coupling of **1** and **2** does not change the stereochemistry of the double bonds in **2**, *E/Z* mixtures of polyenes **2** produced the same mixtures of isomeric products **3**.

This sequence produces the diacids **5** with good overall yields ($\geq 80\%$) and can be applied to the synthesis of analogues with different chain lengths, numbers of conjugated double bonds, or stereochemistries. The key steps in the sequence, namely the cross-coupling followed by the partial reduction with Zn (the acetylene approach) have been previously used with success to obtain arachidonic acid metabolites,^[15] 11-*cis*-retinoids,^[16] and other nonsymmetrical polyenes.^[17]

Compounds **3**, **4**, and **5** are thermally stable for several months if kept in a cool, dark place and their solubility in EtOH or dimethylsulfoxide is in the range of 1–10 μM . These probes have been designed to span typical lipid bilayers, that is, with a maximum expanded length of 35–43 Å,^[18] so that the carboxy groups can reach the two opposite membrane surfaces, and the polyene chromophore is located just in the center of the bilayers. Furthermore, much shorter molecules without saturated spacer chains (not shown) have also been obtained by using the methods described above. In this last case, the electronic interaction between the polyene and the carbonyl end group suppresses the fluorescence.

The large absorption coefficients ($\approx 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) of these bolaamphiphiles facilitate their use in very low concentrations (Figure 2). The fluorescence quantum yields are modest—the highest are those of the compounds with the pentaene group (≈ 0.1). On the other hand, the orientation of the absorption

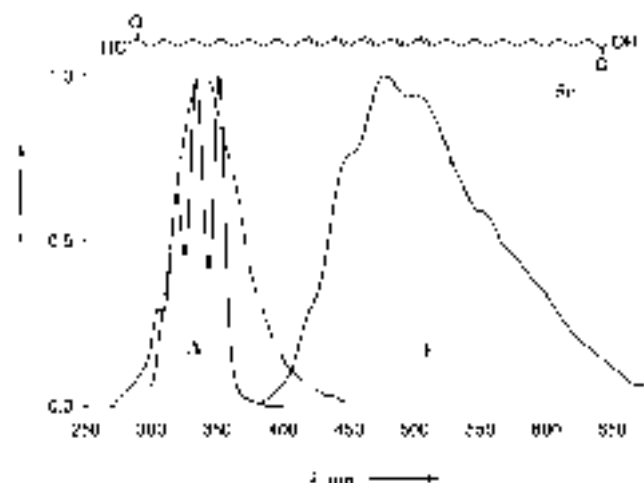


Figure 2. Corrected fluorescence excitation (**A**) and emission (**F**) spectra of the pentaene **5c** in large unilamellar vesicles of DMPC (1,2-dimyristoyl-*sn*-glycerophosphatidylcholine) ($\lambda_{\text{em}} = 468 \text{ nm}$, $\lambda_{\text{ex}} = 347 \text{ nm}$, [**5c**] = 10^{-7} M ; probe/lipid 1:5000). The tryptophan fluorescence (---) from the membrane peptide α -melanocyte-stimulating hormone (α -MSH) in lipid vesicles is overlaid.

and emission transition electronic moments along the long molecular axis is clearly defined and emission takes place in the visible region, thus facilitating the intended applications. The spectra of Figure 2 show the range of absorption and emission wavelengths available from the pentaene diacid **5c** when incorporated into vesicles of pure phospholipids, as well as the large spectral overlap with the tryptophan protein emission, with a donor–acceptor distance at which the transfer efficiency is 50 % (R_0) of $\approx 35 \text{ Å}$. The nonfluorescent bolaamphiphiles that lack the saturated spacer chain present a continuous π -electron cloud that might be of utility as a molecular wire in nanoscale devices.

A key point is the extent to which these probes orientate themselves parallel to the lipid chains in a bilayer. The recently developed polarized two-photon fluorescence microscopy of giant unilamellar vesicles allows the direct observation of the emission transition moment orientation of fluorescence probes in bilayers.^[19] Preliminary experiments in which this technique was applied to the diester probes **4c** and **4d** in the giant unilamellar vesicles of POPC show that the long molecular axis of both compounds is indeed aligned with the phospholipid acyl chains (Figure 3).

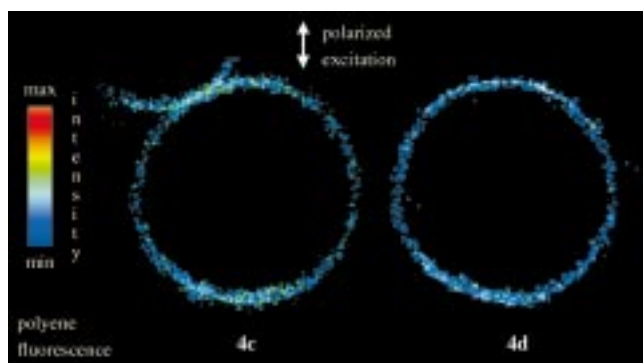


Figure 3. The fluorescence images of giant unilamellar vesicles (diameter 30 μm) of POPC (1-palmitoyl-2-oleoyl-*sn*-glycerophosphatidylcholine) containing the diesters **4c** and **4d** show the two probes aligned preferentially parallel to the phospholipid acyl chains. Two-photon linearly polarized laser excitation (720 nm) oriented as indicated (for details, see ref. [19]).

Experimental Section

1a: Obtained by esterification ($\text{MeOH}/\text{SOCl}_2$) of the corresponding acid; **1b,c**: prepared from 9-dodecyn-1-ol and 3-tetradecyn-1-ol, respectively, by triple bond zipper isomerization to the terminal position,^[20] Jones oxidation to the corresponding carboxylic acid,^[21] and esterification as above ($> 60\%$ overall yield).

(1*E*,3*E*)-**2a**: Obtained from acetylene;^[22] **2b** (1*E*,3*E*,5*E*)/(1*Z*,3*E*,5*E*) 1:4: obtained as described in ref.[23]; **2c** (1*E*,3*E*,5*E*,7*E*)/(1*Z*,3*E*,5*E*,7*E*) 1:4: (2*E*,4*E*)-5-bromo-2,4-pentadienal was treated with triethyl phosphonoacetate in aqueous K_2CO_3 (10 M)^[24] to yield ethyl all-(*E*)-7-bromo-2,4,6-heptatrienoate, which was reduced to the allyl alcohol with DIBAL in toluene/ CH_2Cl_2 at -78°C . The alcohol was then oxidized to the all-(*E*) aldehyde^[23a] with activated MnO_2 . The aldehyde was treated at -50°C with the ylide generated by the treatment of (bromomethyl)triphenylphosphonium bromide with *t*BuOK in THF to give **2c**.

Data of the representative compounds **3c**, **4c**, and **5c**:

3c (*E,E,E*)/(*Z,E,E*) 1:4: Yield 96%; the isomers were separated by precipitation of the all-(*E*) isomer with pentane; (*Z,E,E*)-**3c** is a waxy

oil; all-(*E*)-**3c**: m.p. 76–78 °C (decomp); ¹H NMR (400 MHz, CDCl₃, 25 °C, TMS): δ = 6.50 (ddd, 2H, ³J(H,H) = 7.0 and 15.2, ³J(H,H) = 3.0 Hz; H16, H19), 6.25 (dd, ²J(H,H) = 7.0, ³J(H,H) = 3.0 Hz, 2H; H17, H18), 5.63 (dt, ²J(H,H) = 15.2, ⁴J(H,H) = 2.4 Hz, 2H; H15, H20), 3.66 (s, 6H; 2 × CH₃), 2.33 (dt, ²J(H,H) = 7.2, ⁴J(H,H) = 2.4 Hz, 4H; H12, H23), 2.30 (t, ²J(H,H) = 7.6 Hz, 4H; H2, H32), 1.61 (quint, ²J(H,H) = 7.2 Hz, 4H; H3, H32), 1.52 (quint, ²J(H,H) = 7.6 Hz, 4H; H11, H24), 1.37 (quint, ²J(H,H) = 7.2 Hz, 4H; H10, H25), 1.27 (m, 24H, H4 to H9 and H26 to H31); ¹³C NMR (100 MHz, CDCl₃, 25 °C): δ = 174.3 (2 × CO), 140.1 (C16, C19), 133.2 (C17, C18), 113.1 (C15, C20), 95.2 (C13, C22), 80.2 (C14, C21), 51.4 (2 × CH₃), 34.1 (C2, C33), 29.5, 29.5, 29.4, 29.2, 29.14, 28.9, 28.7 (C4 to C11 and C24 to C31), 24.9 (C3, C32), 19.8 (C12, C23); FT-IR (KBr) (only strong bands): $\tilde{\nu}$ = 2919, 2848, 2222, 1741, 1167 cm⁻¹; UV/Vis (CHCl₃): λ_{max} (ϵ) = 312.0 (45 000), 326.5 (74 000), 343.0 nm (77 000); MS (70 eV): *m/z* (%): 552 (90) [*M*⁺], 521 (17), 353 (29), 339 (22), 183 (32), 169 (62), 155 (100), 141 (63), 129 (63), 117 (29), 105 (15).

4c: A methanolic solution (0.5 mL) of the isomeric mixture of **3c** (0.10 mmol) was treated at room temperature in MeOH/water 1:1 (3 mL) with a slurry of freshly activated Zn powder (9 mmol, < 10 μm).^[14] After 24 h, the subsequent work-up afforded a 1:4 mixture of the pentaene isomers (*Z,E,E,E,Z*) and (*Z,Z,E,E,Z*) in 95% yield. The pure (*Z,E,E,E,Z*) isomer was obtained by using all-(*E*)-**3c**. The isomeric mixture (0.1 mmol) in hexane (200 mL) was heated at reflux for 15 min with iodine-saturated hexane (60 μL) and the solution was filtered through silica gel while hot. The solvent was evaporated to 5 mL and **4c** (all-(*E*) isomer) was separated from the cooled (–20 °C) residual solution by filtration in 95% yield. Highly pure samples were obtained by crystallization from acetone at –20 °C. M.p. 86–89 °C (decomp); ¹H NMR (400 MHz, CDCl₃, 30 °C, TMS): δ = 6.19 (m, 6H; H15 to H20), 6.05 (m, 2H; H14, H21), 5.69 (dt, ²J(H,H) = 7.2 and 14.2 Hz, 2H; H13, H22), 3.66 (s, 6H; 2 × CH₃), 2.29 (t, ²J(H,H) = 7.5 Hz, 4H; H2, H33), 2.09 (dt, ²J(H,H) = 7.2 and 7.2 Hz, 4H; H12, H23), 1.60 (quint, ²J(H,H) = 7.4 Hz, 4H; H3, H32), 1.37 (quint, ²J(H,H) = 7.2 Hz, 4H; H11, H24), 1.25 (brs, 28H; H4 to H10 and H25 to H31); ¹³C NMR (100 MHz, CDCl₃, 30 °C): δ = 174.3 (2 × CO), 135.8 (C13, C22), 133.0, 132.4, 130.9, 130.6 (C14 to C21), 51.4 (2 × CH₃), 34.1 (C2, C33), 32.9 (C12, C23), 29.55, 29.47, 29.43, 29.3, 29.24, 29.20, 29.16 (C4 to C11 and C24 to C31), 25.0 (C3, C32); IR (KBr) (only strong bands): $\tilde{\nu}$ = 3400, 2970, 2880, 2705, 1720, 1420, 1155, 970 cm⁻¹; UV/Vis (CHCl₃): λ_{max} (ϵ) = 305.8 (29 000), 319.8 (60 000), 335.1 (95 000), 353.1 nm (92 000); MS (70 eV): *m/z* (%): 556 (100) [*M*⁺], 525 (11), 357 (19), 343 (45), 145 (32), 131 (60), 117 (65), 105 (39), 91 (51).

5c: Yield 95%, purified by crystallization in toluene at –20 °C; m.p. 141–143 °C (decomp); ¹H NMR (300 MHz, [D₆]DMSO, 70 °C, TMS): δ = 6.22 (m, 6H; H15 to H20), 6.10 (m, 2H; H14, H21), 5.72 (dt, ²J(H,H) = 7.2 and 13.8 Hz, 2H; H13, H22), 2.17 (t, ²J(H,H) = 6.9 Hz, 4H; H2, H33), 2.07 (dt, ²J(H,H) = 6.3 and 6.3 Hz, 4H; H12, H23), 1.50 (quint, ²J(H,H) = 6.9 Hz, 4H; H3, H32), 1.36 (quint, ²J(H,H) = 6.9 Hz, 4H; H11, H24), 1.25 (brs, 28H; H4 to H10 and H25 to H31); ¹³C NMR (75 MHz, [D₆]DMSO, 70 °C): δ = 173.8 (2 × CO), 134.8 (C13, C22), 132.5, 132.0, 130.5, 130.3 (C14 to C21), 33.4 (C2, C33), 31.5 (C12, C23), 28.5 (× 2), 28.44, 28.40, 28.37, 28.3, 28.2 (C4 to C11 and C24 to C31), 24.1 (C3, C32); IR (KBr) (only strong bands): $\tilde{\nu}$ = 3390, 2880, 2710, 1690, 1450, 980, 695 cm⁻¹; UV/Vis (CHCl₃): λ_{max} (ϵ) = 305.8 (26 000), 319.8 (53 000), 335.1 (88 000), 353.1 nm (85 000); MS (70 eV): *m/z* (%): 528 (95) [*M*⁺], 343 (13), 329 (44), 145 (37), 131 (82), 117 (100), 105 (62), 91 (93).

The length of dicarboxylic acids **5** was estimated with MOPAC93 (Chem3D Pro4.5 program, Cambridge Soft) by minimizing the energy with MM2 and AM1 successively down to a gradient of < 0.01 kcal mol⁻¹ Å⁻¹. Solvent effects were not taken into account. Distance OH–OH [Å]: **5a** 40.5, **5b** 35.0, **5c** 42.5, **5d** 37.1, **5e** 40.2.

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- [1] See for example: a) P. Yeagle, *The Membrane of Cells*, Academic Press, Orlando, **1987**; b) A. Kusumi, Y. Sako, *Curr. Opin. Cell Biol.* **1996**, *8*, 566–574; c) “In Search of a New Membrane Model”: *Biol. Skr. K. Dan. Vidensk. Selsk.* **1998**, *49*; d) A. Pralle, P. Keller, E. L. Florin, K. Simons, J. K. Horber, *J. Cell Biol.* **2000**, *148*, 997–1008.

- [2] a) L. M. Louva, A. Fedorov, M. Prieto, *Biophys. J.* **1996**, *71*, 1823–1836; b) J. Szollosi, S. Damjanovich, L. Matyus, *Cytometry* **1998**, *34*, 159–179; c) S. Yegueswaran, M. D. Smirnov, O. Safa, N. L. Esmon, C. T. Esmon, A. E. Johnson, *J. Biol. Chem.* **1999**, *274*, 5462–5468; d) N. M. Hooper, *Mol. Membr. Biol.* **1999**, *16*, 145–156.
- [3] a) A. A. Souto, A. U. Acuña, F. Amat-Guerri, *Tetrahedron Lett.* **1994**, *35*, 5907–5910; b) C. Reyes Mateo, A. A. Souto, F. Amat-Guerri, A. U. Acuña, *Biophys. J.* **1996**, *71*, 2177–2191.
- [4] a) A. Gulik, V. Luzzati, M. De Rosa, A. Gambacorta, *J. Mol. Biol.* **1985**, *182*, 131–149; b) M. Kates, *Membrane Lipids of Archaea*, Elsevier, Oxford, **1993**, pp. 261–295.
- [5] G. H. Escamilla, G. R. Newkome, *Angew. Chem.* **1994**, *106*, 2013–2016; *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1937–1940.
- [6] J. H. Furchop, H. Hungerbühler, U. Siggel, *Langmuir* **1990**, *6*, 1295–1300.
- [7] J. H. Furchop, M. Krull, A. Schulz, D. Möbius, *Langmuir* **1990**, *6*, 497–505.
- [8] a) Y. L. Diyizou, A. Genevois, T. Lazrak, G. Wolff, Y. Nakatani, G. Ourisson, *Tetrahedron Lett.* **1987**, *28*, 5743–5746; b) M. Yamamoto, W. Warnock, A. Milon, Y. Nakatani, G. Ourisson, *Angew. Chem.* **1993**, *105*, 302–304; *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 259–261; c) M. Yamamoto, V. Dollé, W. Warnock, Y. Diyizou, M. Yamada, Y. Nakatani, G. Ourisson, *Bull. Soc. Chim. Fr.* **1994**, *131*, 317–329; d) Y. Nakatani, M. Yamamoto, Y. Diyizou, W. Warnock, V. Dollé, W. Hahn, A. Milon, G. Ourisson, *Chem. Eur. J.* **1996**, *2*, 129–138.
- [9] a) J. M. Delfino, C. J. Stankovic, S. L. Schreiber, F. M. Richards, *Tetrahedron Lett.* **1987**, *28*, 2323–2326; b) J. M. Delfino, S. L. Schreiber, F. M. Richards, *J. Am. Chem. Soc.* **1993**, *115*, 3458–3474.
- [10] a) J. P. Starck, Y. Nakatani, G. Ourisson, *Tetrahedron* **1995**, *51*, 2629–2638; b) P. Starck, Y. Nakatani, G. Ourisson, D. J. Cowley, G. Duportail, *New J. Chem.* **1996**, *20*, 1293–1299.
- [11] E. Quesada, M. Ardhammar, B. Nordén, M. Miesch, G. Duportail, Y. Bounzi-Coulbaly, Y. Nakatani, G. Ourisson, *Helv. Chim. Acta* **2000**, *83*, 2464–2476.
- [12] J. Karolin, S.-T. Bogen, L. B.-Å. Johanson, J. G. Molotkovsky, *J. Fluoresc.* **1995**, *5*, 279–284.
- [13] a) K. Sonogashira, Y. Tohda, N. Hagihara, *Tetrahedron Lett.* **1975**, *16*, 4467–4470; b) S. Takahashi, Y. Kuroyama, K. Sonogashira, N. Hagihara, *Synthesis* **1980**, 627–630; for reviews, see: c) I. B. Campbell in *Organocopper Reagents* (Ed.: R. J. K. Taylor), IRL Press, Oxford, **1994**, pp. 217–235; d) K. Sonogashira in *Metal-catalyzed Cross-coupling Reactions* (Eds.: F. Diederich, P. J. Stang), Wiley-VCH, Weinheim, **1998**, pp. 203–229.
- [14] a) W. Boland, N. Schroer, C. Sieler, M. Eigel, *Helv. Chim. Acta* **1987**, *70*, 1025–1040; b) W. Boland, S. Pantke, *J. Prakt. Chem.* **1994**, *336*, 714–715.
- [15] a) M. Avignon-Tropis, J. R. Pougny, *Tetrahedron Lett.* **1989**, *30*, 4951–4952; b) M. Avignon-Tropis, M. Treilhou, J. R. Pougny, *Tetrahedron* **1991**, *47*, 7279–7286; c) S. Gueugnot, M. Alami, G. Linstrumelle, L. Mambu, Y. Ettit, M. Larchevêque, *Tetrahedron* **1996**, *52*, 6635–6646; d) M. Alami, B. Crouse, G. Linstrumelle, L. Mambu, M. Larchevêque, *Tetrahedron: Asymmetry* **1997**, *53*, 2949–2958.
- [16] B. Borhan, M. L. Souto, J. M. Um, B. Zhou, K. Nakanishi, *Chem. Eur. J.* **1999**, *5*, 1172–1175.
- [17] a) M. Alami, B. Crouse, G. Linstrumelle, *Tetrahedron Lett.* **1994**, *35*, 3543–3544; b) B. Crouse, M. Alami, G. Linstrumelle, *Tetrahedron Lett.* **1995**, *36*, 4245–4248.
- [18] J. F. Nagle, S. Tristram-Nagle, *Curr. Opin. Struct. Biol.* **2000**, *10*, 474–480.
- [19] L. A. Bagatolli, E. Gratton, *Biophys. J.* **2000**, *78*, 290–305.
- [20] F. Menger, S. Brocchini, X. Chen, *Angew. Chem.* **1992**, *104*, 1542–1543; *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 1492–1493.
- [21] a) S. Shak, N. O. Reich, I. M. Goldstein, P. R. Ortiz de Montellano, *J. Biol. Chem.* **1985**, *260*, 13023–13028; b) N. Hébert, A. Beck, R. B. Lennox, G. Just, *J. Org. Chem.* **1992**, *57*, 1777–1783.
- [22] S. A. Mitchenko, V. P. Ananikov, I. P. Veletskaya, Y. A. Ustynuk, *Mendeleev Commun.* **1997**, 130–131.
- [23] a) D. Soulez, G. Plé, L. Duhamel, P. Duhamel, *J. Chem. Soc. Chem. Commun.* **1995**, 563–564; b) D. Soulez, G. Plé, L. Duhamel, *J. Chem. Soc. Perkins Trans. 1* **1997**, 1639–1645.
- [24] J. Villeras, M. Rambaud, *Synthesis* **1983**, 300–303.