To our knowledge this is the first report on the use of single-molecule atomic-force spectroscopy to study the reduction pathway of multiple disulfide bonds in proteins and to evaluate the distributions of intermediates obtained under different reducing conditions without separating them and without any blocking and fractionation steps. The characterization of these intermediates has so far been accomplished by first blocking them with reagents such as alkylalkanethiosulfonates and then by fractionation by ion-exchange chromatography, 2D or capillary gel electrophoresis, or gel filtration.[11] The determination of thiol groups and disulfide bonds in a polythiol systems has always been a very challenging problem.[12]

The single-molecule force-spectroscopy data presented here show: 1) how a redox environment can modulate the mechanical properties of angiostatin; 2) how this modulation relies, at the single-molecule level, on the extent of reduction of the disulfide bonds; and 3) how, at the level of a large sample of molecules, the distribution of the different thiol/disulfide intermediates after reduction can be estimated by statistical analysis of the force curves.

Experimental Section

Ang(1–5), purchased from Calbiochem with purity greater than 95%, was adsorbed onto a Petri dish from a 20–100 μgmL PBS (pH 7.4) buffer solution for 3–5 min and then rinsed with the same buffer. The Petri dish was previously cleaned and exposed to high-energy nitrogen plasma for 15 min. The force-spectroscopy experiments were performed with a Nanoscope IIIa, Digital Instrument, Santa Barbara, CA, with silicon nitride (Si_3N_4) probes (Microlevers, Thermomicroscopes) with a spring constant of 40–55 $\text{pN}\,\text{nm}^{-1}$, determined by a thermal oscillation method.[13] A velocity of 1 $\mu\text{m}\,\text{s}^{-1}$ during tip approach and retraction, a loading force of about 1 nN, and a contact time between the tip and the surface of 200 ms were used. We started recording the force curves 30 min after injection of 5 or 50 mM DTT (Aldrich), and 60 min after injection of 100mM DTT into the fluid cell.

The same experiment was repeated with the following two modifications: First, after reduction with 5, 50, or 100 mM DTT, we injected a 100 mM solution of iodoacetamide (Fluka) into the fluid cell, and allowed it to stand for 20 min. Second, the iodoacetamide solution was subsequently replaced with PBS, under which the force-spectroscopy measurements were then carried out.

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[1] a) M. S. O'Reilly, L. Holmgren, Y. Shing, C. Chen, R. A. Rosenthal, M. Moses, W. S. Lane, Y. Cao, E. H. Sage, J. Folkman, *Cell* **1994**, *79*, 315; b) A. M. Mulichak, A. Tulinsky, K. G. Ravichandran, *Biochemistry* **1991**, *30*, 10576.

[2] a) M. Conti, Y. Bustanji, G. Falini, P. Ferruti, S. Stefoni, B. Samorì, *ChemPhysChem* **2001**, *2*, 610; b) M. Conti, G. Donati, G. Cianciolo, S. Stefoni, B. Samorì, *J. Biomed. Mater. Res.*, in press.

[3] M. Rief, M. Gautel, F. Oesterhelt, J. M. Fernandez, H. E. Gaub, *Science* **1997**, *276*, 1109.

[4] M. Grandbois, M. Beyer, M. Rief, H. Clausen-Schaumann, H. E. Gaub, *Science* **1999**, *283*, 1727.

[5] P. Carl, C. H. Kwok, G. Manderson, D. W. Speicher, D. E. Discher, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 1565.

[6] T. E. Fisher, P. E. Marszalek, J. M. Fernandez, *Nat. Struct. Biol.* **2000**, *7*, 719.

[7] C. Bustamante, J. F. Marko, E. D. Siggia, S. Smith, *Science* **1994**, *265*, 1599.

[8] W. Konigsberg, *Methods Enzymol.* **1972**, *25*, 185.

[9] J. Y. Chang, *J. Biol. Chem.* **1997**, *272*, 69.

[10] a) M. Trexler, L. Patthy, *Biochim. Biophys. Acta* **1984**, *787*, 275; b) M. Trexler, L. Patthy, *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 2457.

[11] a) W. J. Wedemeyer, E. Welker, M. Narayan, H. A. Scheraga, *Biochemistry* **2000**, *39*, 4207; b) T. E. Creighton, *Methods Enzymol.* **1986**, *131*, 83.

[12] A. Fontana, C. Toniolo in *The Chemistry of The Thiol Group, Part 1* (Ed: S. Patai), Wiley, London, **1974**, p. 271.

[13] E. L. Florin, M. Rief, H. Lehmann, C. Ludwig, V. T. Dornmair, V. T. Moy, H. E. Gaub, *Biosens. Bioelectron.* **1995**, *10*, 895.

Colorimetric and Fluorometric Detection of Nucleic Acids Using Cationic Polythiophene Derivatives**

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Simple and reliable sequence-specific methods are needed for the rapid detection of oligonucleotides, to diagnose infections and various genetic diseases. In this regard, interesting optical and electrochemical DNA-hybridization sensors have been proposed.[1–5] The recognition capabilities of DNA are well established but, to transduce the recognition event into a physically measurable value, a fluorescent or electroactive tag is often bound to the analyte. Electrochemical and optical sensors based on conjugated polymers have also been reported[6–9] and some oligonucleotide-functionalized conjugated polymers can also transduce hybridization events into an electrical signal without labeling of the oligonucleotide target.[10–12] The detection relies on a mod-

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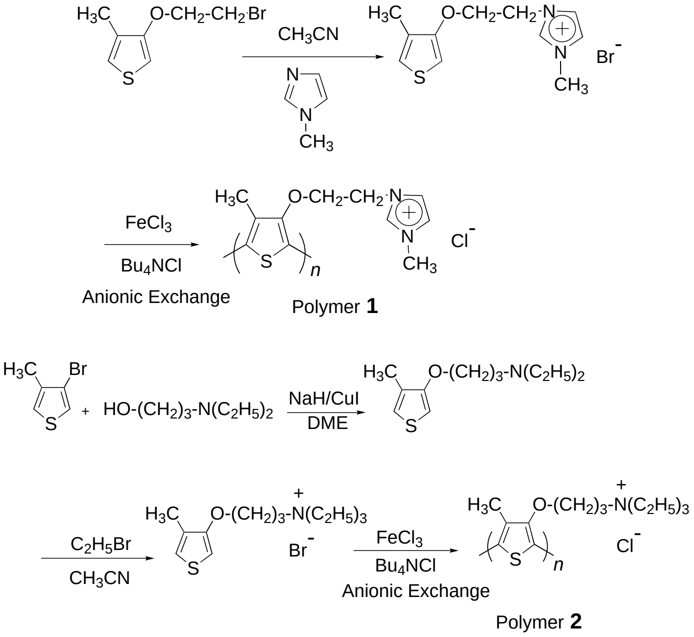
ification of the electrical properties of the conjugated polymers, through the capture of the complementary oligonucleotides. An assay that does not require any chemical manipulation of nucleic acids or complex reaction mixtures would be greatly advantageous.

We report here new water-soluble cationic polythiophene derivatives, which can easily transduce oligonucleotide hybridization with a specific 20-mer capture probe into a clear optical (colorimetric or fluorometric) output. This simple, rapid, and versatile methodology does not require any chemical reaction of the probes or the analytes; it is based on different electrostatic interactions and conformational structures between electroactive and photoactive cationic poly(3-alkoxy-4-methylthiophene)s, and single-stranded oligonucleotides or double-stranded (hybridized) nucleic acids.

We prepared the cationic, water-soluble, electroactive, and photoactive polymers **1** and **2** (Scheme 1). The new monomers were obtained using straightforward reactions[13–16] and the related polymers were prepared from an oxidative polymerization in chloroform, by using $FeCl_3$ as the oxidizing agent.[17] As reported for anionic poly(3-alkoxy-4-methylthiophene)s,[17, 18] this method of polymerization usually yields well-defined polymers with a number-average molecular weight of about 6–10 kDa and a polydispersity index of about 1.2–2.9. However, as recently reported by Lukkari et al.[18] for similar cationic polythiophenes, attempts to determine the molecular weight of the present polythiophenes by size-exclusion chromatography (SEC) experiments were not successful, presumably because SEC columns contain some anionic groups, which lead to a strong adsorption of these cationic polymers. Matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) spectroscopic measurements were also unsuccessful.



Scheme 1. Synthesis of polymers **1** and **2**. Experimental procedures and characterization data are in the Supporting Information.

As expected, the resulting polymers **1** and **2** are soluble in aqueous solutions. For instance, at 55 °C, aqueous solutions (0.1 M NaCl or 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer/0.1 M NaCl) of the cationic polymer **1** are yellow ($\lambda_{max} = 397$ nm; Figure 1 A,a and B,a). This absorption maximum at a short wavelength is related to a random-coil conformation of the polythiophene derivative, as any twisting of the conjugated backbone leads to a decrease in the effective conjugation length[6] (Figure 2). As with any water-soluble cationic polyelectrolytes, these polythiophene deriv-

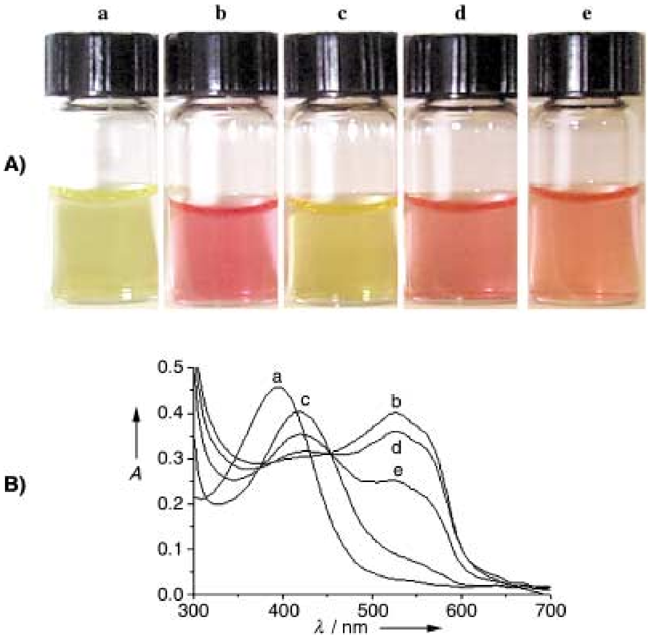


Figure 1. A) Photographs of solutions (7.9×10^{-5} M, on a monomeric unit basis) of a) polymer **1**, b) polymer **1/X1** duplex, c) polymer **1/X1/Y1** triplex, d) polymer **1/X1/Y2** mixture, and e) polymer **1/X1/Y3** mixture after 5 min of mixing at 55 °C in 0.1 M NaCl/H_2O. B) UV/Vis absorption spectra corresponding to the different assays in photograph A.

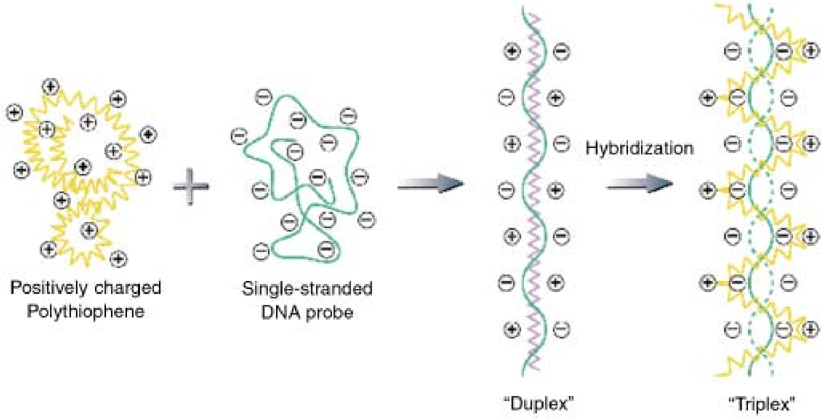


Figure 2. Schematic description of the formation of polythiophene/single-stranded nucleic acid duplex and polythiophene/hybridized nucleic acid triplex forms.

atives can form strong complexes with negatively charged oligomers and polymers.[19] We report here the use of three types of negatively charged oligonucleotides: a capture-probe sequence (**X1**: 5′-CATGATTGAACCATCCACCA-3′), a perfect complementary target (**Y1**: 3′-GTACTAACTTGG-TAGGTGGT-5′), a two-mismatch complementary target (**Y2**: 3′-GTACTAACTT*CGA*AGGTGGT-5′), and a one-mismatch complementary target (**Y3**: 3′-GTACTAACTT*C*G-TAGGTGGT-5′). The oligonucleotides **X1** and **Y1** are a specific oligonucleotide pair for *Candida albicans*, **Y2** is a specific oligonucleotide for *Candida dubliniensis*, and **Y3** is a variant designed to have single mismatches with either *C. albicans* or *C. dubliniensis* sequences. Upon addition of 1.0 equivalent, on a monomer unit basis (the concentrations of the 20-mer oligonucleotides are therefore a twentieth of those reported for the synthetic polydisperse polythiophene, which are given per repeat unit), of capture oligonucleotide **X1**, the mixture becomes red ($\lambda_{\max} = 527$ nm) within 5 min (Figure 1 A,b and B,b), because of the formation of a so-called duplex between the polythiophene and the oligonucleotide probe (see Figure 2). However, it is difficult to determine whether these duplexes are isolated species or form aggregates. Precipitation occurs after several hours. After 5 min of mixing in the presence of 1.0 equivalent of the complementary oligonucleotide **Y1**, the solution becomes yellow ($\lambda_{\max} = 421$ nm) (Figure 1 A,c and B,c); presumably caused by the formation of a new complex termed a triplex (Figure 2), formed by complexation of the polymer with the hybridized nucleic acids. No precipitation was observed, even after several hours. Further addition of 1.0 equivalent of the probe oligonucleotide **X1** does not induce any color change, either in the duplex or in the triplex. The same is true for the addition of another 1.0 equivalent of the target oligonucleotide **Y1** to the triplex. These results highlight the stronger affinity of the cationic polymer towards double-stranded oligonucleotides. It is also important to note that duplexes are unstable at temperatures higher than 65 °C, whereas triplexes do not form at temperatures lower than 45 °C. Very similar results have been obtained with different probe and target oligonucleotides and with polymer **2**.

On the basis of previous studies performed on thermochromic, solvatochromic, and affinitychromic (changing color upon binding) regioregular poly(3-alkoxy-4-methylthiophene)s,[6] it is believed that these colorimetric effects are made possible because of a different conformational structure of the conjugated polymer in the duplex form (highly conjugated, planar conformation) compared to that observed in the triplex form (less conjugated, nonplanar conformation; Figure 2). In addition, as shown in Figure 3, circular-dichroism (CD) measurements did not reveal any optical activity for polymer **1** in its random coil and duplex forms, but showed a bisignate CD spectrum centered at 420 nm in the triplex form, characteristic of a right-handed helical orientation of the polythiophene backbone.[9] Such a right-handed helical structure is compatible with the binding of the polymer to the negatively charged phosphate backbone of DNA. The presence of a CD signal only in the spectrum of the triplex form, combined with the presence of a clear isosbestic point as a function of the amount of the complementary strand (not shown here), indicates the coexistence of only two distinct conformational structures (triplex and duplex) for the polythiophene. In addition, these measurements indicate that triplexes are stable up to 80 °C. This difference in thermal stability between duplexes and triplexes could be extremely useful for the future design of solid-state sensors where the probes could be covalently attached to different substrates.

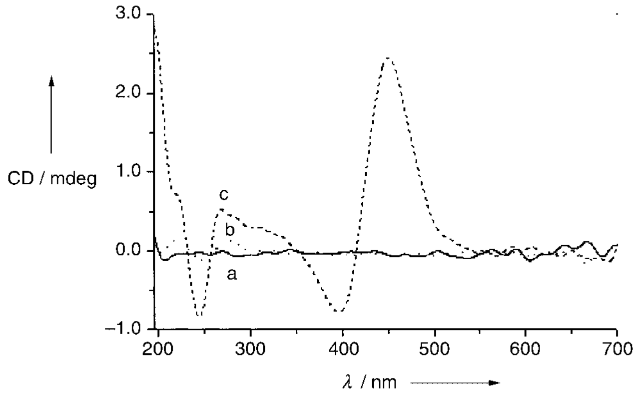


Figure 3. Circular dichroism spectrum of a solution (1.2×10^{-4} M, on a monomer unit basis) of a) polymer **1**, of b) polymer **1/X1** duplex, and c) polymer **1/X1/Y1** triplex, at 55 °C, in 10 mM Tris-buffered aqueous solution containing 0.1 M NaCl (pH 8).

To verify the specificity of this polymeric optical transducer in the presence of imperfect or incomplete hybridizations, different oligonucleotides (20-mers differing by only 1 or 2 nucleotides) were investigated. A very distinct, stable (up to several hours), and reproducible UV/Vis absorption spectrum is observed in the case of oligonucleotide targets with two mismatches (**Y2**; Figure 1 B,d) when compared to perfect hybridization (Figure 1 B,c). It is also possible to distinguish only one mismatch (Figure 1 A,e and B,e). In the case of one mismatch, the complexation kinetics were monitored; after 30–60 min at 55 °C, the color changed back to yellow. However, it is possible to stop the color change after 5 min by cooling the solution to room temperature. Therefore, it is easy to distinguish one or two mismatches from no mismatch in 5 min. Once again, it is interesting to note that the spectra of duplex and triplex forms show an isosbestic point, which indicates the presence of only two distinct conformational structures for the polymeric transducer. The detection limit of this colorimetric method is about 1×10^{13} molecules of oligonucleotide (20-mers), in a total volume of 100 μL (which is a concentration of 2×10^{-7} M).

A fluorometric detection of oligonucleotide hybridization is also possible since the fluorescence of poly(3-alkoxy-4-methylthiophene)s is quenched in the planar, aggregated form.[6] At 55 °C, the yellow form of polymer **1** is fluorescent (quantum yield of 0.03, measured against polythiophene standards,[6] with a maximum of emission at 530 nm, Figure 4,a) but upon addition of 1.0 equivalent of a negatively charged capture oligonucleotide probe **X1**, the fluorescence intensity decreases and the maximum of emission is slightly red-shifted (Figure 4,b). When hybridization with the complementary strand **Y1** takes place, the formation of a polymeric triplex leads to a fivefold rise in fluorescence intensity (Figure 4,c). Interestingly, upon addition of one (Figure 4,d)

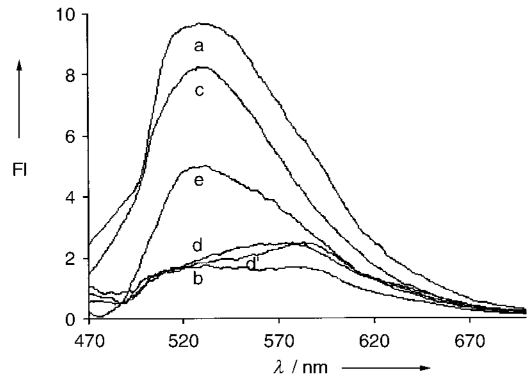


Figure 4. Fluorescence spectrum (FI = fluorescence intensity) of a solution (2.0×10^{-7} M, on a monomer unit basis) of a) polymer **1**, b) polymer **1/X1** duplex, c) polymer **1/X1/Y1** triplex, d) polymer **1/X1/Y2** mixture, d′) polymer **1/X1/Y2** (100 equivalents) mixture and e) polymer **1/X1/Y3** mixture at 55 °C, in 10 mM Tris buffer containing 0.1M NaCl (pH 8).

or even 100 equivalents (Figure 4,d′) of the target oligonucleotide with two mismatches (**Y2**), the fluorescence intensity is not significantly modified. It is even possible to distinguish oligonucleotides with one mismatch (Figure 4,e). By measuring the fluorescence intensity at 530 nm (without recording the entire emission spectrum), it is possible to detect the presence of as few as 3×10^6 molecules of the perfect complementary oligonucleotide (20-mers) in a volume of 200 μL (this is a concentration of 2×10^{-14} M). Moreover, covalent attachment of the oligonucleotide to the fluorescent conjugated polymer, or use of an optimized fluorescence detection procedure based on a high-intensity blue diode (as the excitation source) and a nondispersive, interference filter-based system should yield even more sensitive and more specific detection capability. The electroactivity of these cationic polythiophene derivatives in aqueous solutions could also be exploited for the detection of DNA-hybridization events.

In conclusion, a novel methodology that allows simple optical (colorimetric or fluorometric) detection of nucleic acids has been developed. This rapid, selective, sensitive (as few as 3×10^6 molecules of oligonucleotide in 200 μL can be detected), and versatile method does not require any chemical reaction of the probes or the analytes and is based on conformational modifications of the conjugated backbone of cationic poly(3-alkoxy-4-methylthiophene)s, when mixed with single-stranded or double-stranded (hybridized) oligonucleotides. This procedure could provide inexpensive methodologies for the rapid detection and identification of nucleic acids.

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[1] S. P. A. Fodor, J. L. Read, M. C. Pirrung, L. Stryer, A. T. Lu, D. Solas, *Science* **1991**, *251*, 767–773.
[2] T. Livache, A. Roget, E. Dejean, C. Barthet, G. Bidan, R. Teoule, *Nucleic Acids Res.* **1994**, *22*, 2915–2921.
[3] S. Tyagi, F. R. Kramer, *Nat. Biotechnol.* **1996**, *14*, 303–308.
[4] S. R. Mikkelsen, *Electroanalysis* **1996**, *8*, 15–19.
[5] T. A. Taton, C. A. Mirkin, R. L. Letsinger, *Science* **2000**, *289*, 1757–1760.
[6] M. Leclerc, *Adv. Mater.* **1999**, *11*, 1491–1498.
[7] D. T. McQuade, A. E. Pullen, T. M. Swager, *Chem. Rev.* **2000**, *100*, 2537–2574.
[8] L. Chen, D. W. McBranch, H. L. Wang, R. Hegelson, F. Wudl, D. G. Whitten, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 12287–12292.
[9] P. C. Ewbank, G. Nuding, H. Suenaga, R. D. McCullough, S. Shinkai, *Tetrahedron Lett.* **2001**, *42*, 155–157.
[10] H. K. Youssoufi, F. Garnier, P. Srivastava, P. Godillot, A. Yassar, *J. Am. Chem. Soc.* **1997**, *119*, 7388–7389.
[11] P. Bäuerle, A. Emge, *Adv. Mater.* **1998**, *10*, 324–330.
[12] H. K. Youssoufi, A. Yassar, *Biomacromolecules* **2001**, *2*, 58–64.
[13] K. Faïd, M. Leclerc, *Chem. Commun.* **1996**, 2761–2762.
[14] P. Lucas, N. E. Mehdi, A. H. Ho, D. Bélanger, L. Breau, *Synthesis* **2000**, *9*, 1253–1258.
[15] A. El Kassmi, G. Héraud, W. Büchner, F. Fache, M. Lemaire, *J. Mol. Catal.* **1992**, *72*, 299–305.
[16] P. B. Balanda, M. B. Ramey, J. R. Reynolds, *Macromolecules* **1999**, *32*, 3970–3978.
[17] M. Chayer, K. Faïd, M. Leclerc, *Chem. Mater.* **1997**, *9*, 2902–2905.
[18] J. Lukkari, M. Salomäki, A. Viinikanoja, T. Ääritalo, J. Paukkunen, N. Kocharova, J. Kankare *J. Am. Chem. Soc.* **2001**, *123*, 6083–6091.
[19] T. K. Bronich, H. K. Nguyen, A. Eisenberg, A. V. Kabanov, *J. Am. Chem. Soc.* **2000**, *122*, 8339–8343.

Conformationally Flexible, Chiral Quaternary Ammonium Bromides for Asymmetric Phase-Transfer Catalysis[**]

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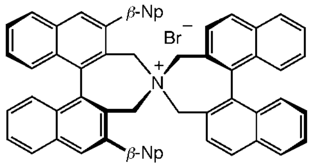
As clearly demonstrated by a number of notable recent successes, the use of phase-transfer catalysis for the preparation of chiral, nonracemic organic compounds from prochiral substrates using chiral catalysts such as optically pure quaternary ammonium salts has become a field of growing importance.[1, 2] We recently contributed to this area by introducing chiral C_2-symmetric quaternary ammonium bromides of type **1** which efficiently catalyze the phase-transfer



1 [(*S*,*S*)-β-Np-NAS-Br]

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