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A New C_2-Symmetric Atropisomeric Thiophene-Based Monomer for Inherently Chiral Electroactive Materials: Synthesis, HPLC Resolution, and Absolute Configuration Assignment

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Abstract: Herein, we report on the synthesis and high-performance liquid chromatography (HPLC) resolution of a new atropisomeric C_2-symmetry chiral monomer based on the 3,3′-bithiophene core, which was developed to produce novel, inherently oligomeric chiral electroactive materials. The analytical enantioseparation was optimized using the cellulose-type Chiralpak IB-3 column and a mixture of n-hexane–methanol–dichloromethane 90:5:5 (*v*/*v*/*v*) as the mobile phase. During the scale-up of the enantioseparation analytical conditions to a semipreparative level, remarkable deformations in the HPLC profile, such as peak splitting and plateau zones between enantiomeric peaks, were observed. We demonstrate the effects of sample diluent as they relate to distorted peak profiles, as well as provide experimental solutions to prevent the disturbing phenomenon. The optimized chromatographic conditions were exploited to collect milligram amounts of the enantiopure sample, which was submitted to chiroptical and stereochemical characterization studies.

Keywords: 3,3′-dibenzothiophene derivative; semi-preparative HPLC; enantioseparation; absolute configuration; electronic circular dichroism

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1. Introduction

Due to their well-established enantioselective properties, inherently chiral linear or cyclic polyheterocycles are used as enantiodiscriminative chiral electrode surfaces [1,2]. The chirality of these materials is not due to the presence of one or more stereogenic centers, but derives from a tailored torsion of the whole main molecular scaffold with rotational barriers too high to be overcome at room temperature. The molecular architecture is such that the stereogenic and the electroactive elements coincide [3].

The production of the enantiomers of chiral synthons with C_2 symmetry and endowed with thiophene pendants involves a semipreparative resolution step based on enantioselective high-performance liquid chromatography (HPLC) on a chiral stationary phase (CSP) and successive oligomerization by oxidation of the collected enantiopure forms [4]. In particular, thiophene α-positions involved in the oxidative coupling are homotopic and the products of oxidation maintain a complete regioregularity. The homochiral oligomer products have the same (*R*) or (*S*) absolute configuration as the starting enantiomer and usually are formed from two to four structural units. When applied as chiral electrode surfaces in electrochemistry experiments, the inherently chiral oligothiophenes have been shown to produce impressive peak potential differences for the enantiomers of chiral probes that are reversed when the absolute configuration of the selector is changed [2,3].

At a microscopic level, chromatographic enantioseparation of the atropisomeric monomers can be achieved when the resolvable enantiomers exploit enantiospecific differences in the interaction with the active sites of the selector used in the CSP [4,5].

Nevertheless, it is noteworthy that the chiral recognition on a given CSP is a dynamic process (i.e., the adsorption of enantiomers onto CSP is a distribution equilibrium between two phases) and, even if thermodynamically favored, it can be hindered or prevented by the onset of competitive processes and equilibria involving the enantiomers. In other words, when a secondary competitive process involving the enantiomers occurs on the time scale required to perform the desired enantiodiscrimination, this latter process could be severely disturbed, or even compromised.

Enantiomerization of stereolabile enantiomers that invert their absolute configuration on the separation time scale is a well-known dynamic on-column process [6]. The peak splitting and/or deformation of peak profiles can also originate from the slow mass transfer, slow equilibration of analytes between stationary and mobile phase, insufficient stationary sites to adsorb analytes (due to overloading), fast self-aggregation, and eluent mismatches [7–9].

In this work, we report on the synthesis of a new chiral monomer for inherently chiral oligothiophenes, namely the compound 2,2′-bis(2,2′:5′,2′′-terthiophen-5-yl)-3,3′-bithianaphthene (abbreviated as **BT2**–**T6**, Figure 1), and its semipreparative HPLC enantioseparation. **BT2**–**T6** belongs to the class of atropisomeric C_2 symmetric thiophene-based electroactive monomers whose progenitor is **BT2**–**T4** [3,10], featuring the same atropisomeric core functionalized in 3 and 3′ positions with 2,2-bithiophenic groups and successfully resolved into antipodes by enantioselective HPLC analysis [11]. The key feature of **BT2**–**T4** relies on the fact that the chemical [12] or electrochemical oxidation of its enantiomers produces inherently chiral films showing outstanding enantiodiscrimination ability, both in chiral voltammetry [13–15] where the antipodes of probes, differing in their functional groups and stereogenic elements, are discriminated through cyclovoltammetry on the basis of the difference in their oxidative potentials. More recently, the enantiopure oligomers of **BT2**–**T4** were exploited in bipolar electrochemistry for the development of an on–off system for the absolute discrimination of applicative probes (e.g., L-DOPA) as well as for the direct dynamic expression of molecular chirality with autonomous swimmers based on enzymes [16–18].

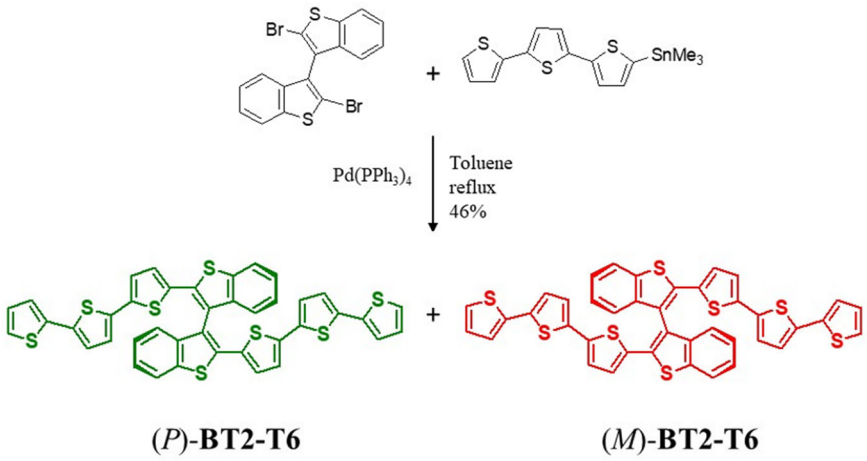

Figure 1. The final step in the synthesis of the racemates of **BT2**–**T6**.

Finally, the chiroptical properties of the enantiomers collected in the optimized HPLC conditions were exhaustively determined and correlated with those of the parent **BT2**–**T4** to determine the absolute configuration of the enantiopure forms.

2. Materials and Methods

2.1. Synthesis

2.1.1. Chemicals and Reagents

All reactions were performed with oven-dried laboratory glassware under a nitrogen atmosphere unless otherwise indicated. All reactants and dry solvents were purchased from Sigma Aldrich (St. Louis, MO, USA), Tokyo Chemical Industry (Chennai, India), and Fluorochem (Hadfield, UK), and were used as received. TLC analyses were performed on ALUGRAM® Xtra SIL G/UV254 (0.2 mm thin layer depth; Macherey-Nagel). Gravimetric chromatography columns were performed using silica gel (particle diameter: 0.63–2.00 mm) as stationary phase. ^{1}H-NMR spectra were recorded with Bruker AMX 300 instrument operating at 300 MHz with TMS as internal standard. ^{13}C-NMR spectra were recorded at 100.56 MHz with total proton decoupling with TMS as internal standard. Low-resolution mass spectra (MS) were recorded on a Thermo-Finnigan LCQ Advantage mass spectrometer (ESI ion source).

2.1.2. Synthesis of 5-(trimethylstannyl)-2,2′:5′,2′′-terthiophene

A 2.5 M *n*-BuLi solution (in *n*-hexane) (1 eq., 2.4 mL, 6 mmol) was added dropwise to a solution of 2,2′:5′,2′′-terthiophene (1 eq., 1.5 g, 6 mmol) in dry THF (35 mL) over a 5-minute period at −78 °C, and the mixture was stirred for 20 min. A trimethyltin chloride (1.1 eq., 1.3 g, 6.6 mmol) solution in dry THF (15 mL) was added, and the mixture was stirred for 1 h at −78 °C and then for 24 h at room temperature. The mixture was quenched with water; the two phases were separated and the aqueous phase was extracted with dichloromethane. The collected organic phases were dried over $MgSO_4$, filtered, and the solvent was removed under reduced pressure to afford the desired product as a brown oil used without any further purification. Yield from ^{1}H NMR: (84%); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 7.30 (m, 1H), 7.24 (m, 1H), 7.19 (m, 1H), 7.05 (d, *J* = 3.4 Hz, 1H), 7.12–7.09 (m, 2H), 7.06–7.03 (m, 1H), 0.27 (t, *J* = 7.0 Hz, 9H).

2.1.3. Synthesis of BT2-T6

5-Trimethylstannyl-2,2′:5′,2′′-terthiophene (2 eq., 4.8 mmol) and $Pd(PPh_3)_4$ (0.05 eq., 260 mg, 0.25 mmol) were added under nitrogen atmosphere to a warm stirred solution of 2,2′-dibromo-3,3-bithianaphthene (1 eq., 980 mg, 2.3 mmol) in dry toluene (45 mL). The mixture was refluxed for 24 h; then, the solvent was removed under reduced pressure to give a residue that was purified by column chromatography (*n*-hexane-dichloromethane 7:3) to afford the desired product as an orange solid.

Yield: 800 mg (46%); M.P.: 194 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.95 (d, *J* = 9.0 Hz, 2H), 7.45–7.38 (m, 2H), 7.31–7.24 (m, 6H), 7.22 (d, *J* = 6 Hz, 2H), 7.18 (dd, *J*1 = 9.0 Hz, *J*2 = 3.0 Hz, 2H), 7.05–7.00 (m, 6H), 6.92 (d, *J* = 3.0 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 140.4, 138.6, 138.3, 136.8, 136.6, 135.3, 134.2, 127.9, 127.4, 125.4, 125.1, 124.7, 124.6, 124.3, 123.8, 123.6, 122.7, 122.1; MS (ESI): 758.0.

The ^{1}H NMR and ^{13}C NMR spectra are shown in Figure S1 of Supplementary Materials.

The final step in the synthesis of racemic **BT2–T6** is shown in Figure 1.

2.2. Enantioselective HPLC

HPLC-grade solvents were supplied by Aldrich (Milan, Italy). HPLC enantioseparations were conducted using stainless-steel columns, Chiralpak® IB-3 100 × 4.6 mm, 3 µm, and Chiralpak® IB 250 mm × 10 mm, 5 µm (Chiral Technologies Europe, Illkirch-Graffenstaden, France). The analytical HPLC apparatus consisted of a PerkinElmer 200 LC pump equipped with a Rheodyne injector, a 50 µL sample loop, an HPLC Dionex CC-100 oven, and a Jasco Model CD 2095 Plus UV/CD detector. For semipreparative resolutions, a PerkinElmer 200 LC pump equipped with a Rheodyne injector, a 5 mL sample loop, a PerkinElmer LC 101 oven, and a Waters 484 detector were employed. The signal was acquired and processed by the Clarity software of DataApex.

2.3. Chiroptical Measurements

The electronic circular dichroism (ECD) spectra of the enantiomers of **BT2**–**T6** collected on a semipreparative scale were recorded in dichloromethane at 25 °C using a Jasco Model J-700 spectropolarimeter. The optical path was 1 mm. The spectra average are computed over four instrumental scans and the intensities are presented in terms of ellipticity values (mdeg).

Specific rotations were measured by a PerkinElmer polarimeter model 241 equipped with Na/Hg lamps. The volume of the cell was 1 mL and the optical path was 10 cm. The temperature was set at 20 °C.

3. Results

3.1. Synthesis

The synthesis of racemic **BT2**–**T6** was planned in accordance with the workflow followed for the preparation of **BT2**–**T4** [3] through a Stille coupling starting from 2,2′-dibromo-3,3′-bithianaphthene and 5-(trimethylstannyl)-2,2′:5′,2″-terthiophene (Figure 1). The reaction was performed in refluxing toluene in the presence of palladium (tetrakis)triphenylphosphine as a catalyst and the targeted compound was obtained as an orange solid in 48% yield after column chromatography. The stannyl derivative was obtained by lithiation of commercially available terthiophene with a 2.5 M n-BuLi solution in dry THF at −78 °C and the corresponding lithium derivative was treated with trimethyltin chloride. The stannyl derivative was used without any further purification in the Stille [19] Pd(0)-catalyzed coupling with the 2,2′-dibromo-3,3-bithianaphthene (the unreacted terthiophene was estimated to be about 10%). The reaction mechanism is based on a catalytic cycle constituted by three steps, namely oxidative addition, transmetallation reaction, and reductive elimination [20].

3.2. HPLC Resolution

The first set of the HPLC tests performed in the course of this work was dedicated to research into enantioselective conditions capable of separating the enantiomers of the atropisomeric compound **BT2**–**T6** on an analytical scale.

Different CSPs (Chiralpak IB-3, Chiralpak IA, Chiralpak IC, and Chiralpak IG) were selected and screened by using low-polarity normal-phase eluents. Non-standard (dichloromethane and acetone) and standard alcoholic (methanol, ethanol, and 2-propanol) solvents in a mixture with n-hexane were investigated in binary and ternary mobile phase systems. The best results in terms of enantioselectivity and analysis times were obtained employing the 100 mm × 4.6 mm Chiralpak IB-3 column and the mixture n-hexane–methanol–dichloromethane 90:5:5 ($v/v/v$) as the mobile phase. Under these conditions, very high enantioselectivity (α = 3.11) and resolution (Rs = 13.03) factor values were achieved within 7 min. In the Chiralpak IB-3 CSP, the cellulose tris(3,5-dimethylphenylcarbamate) polymeric selector is immobilized onto 3 μm silica particles. In the successive step of optimization of the enantioselective method, a temperature-dependent study was performed. The enantioseparation and resolution factors were calculated between 15 °C and 35 °C in 10 °C increments. As can be seen in Figure 2, the enantioseparation process occurred within the enthalpy-controlled domain [21] and the enantioselectivity decreased as the temperature increased. By fixing the column temperature at 15 °C, the enantioseparation and resolution factor values of **BT2**–**T6** increased to 4.07 and 19.13, respectively, (Figure 2) without excessively extending the analysis time (less than 10 min).

Comparing these chromatographic parameters with those obtained by analyzing the analog **BT2**–**T4** in the same conditions (i.e., 2.47 and 8.95, respectively), it is evident that the introduction of a third thiophene moiety in the wings of the atropisomeric scaffold duplicated the enantioseparation and resolution factor values.

After developing the enantiomer separation method on a small scale, the next step of our work was to transfer it to a larger scale and isolate both enantiomers in multimilligram amounts. By taking into account appropriate scale factors and using semipreparative

columns containing the same packing material as the analytical ones, the scale-up process is typically easily accomplished, especially when the values of enantioseparation and resolution are as high as those observed for **BT2–T6** on the Chiralpak IB CSP.

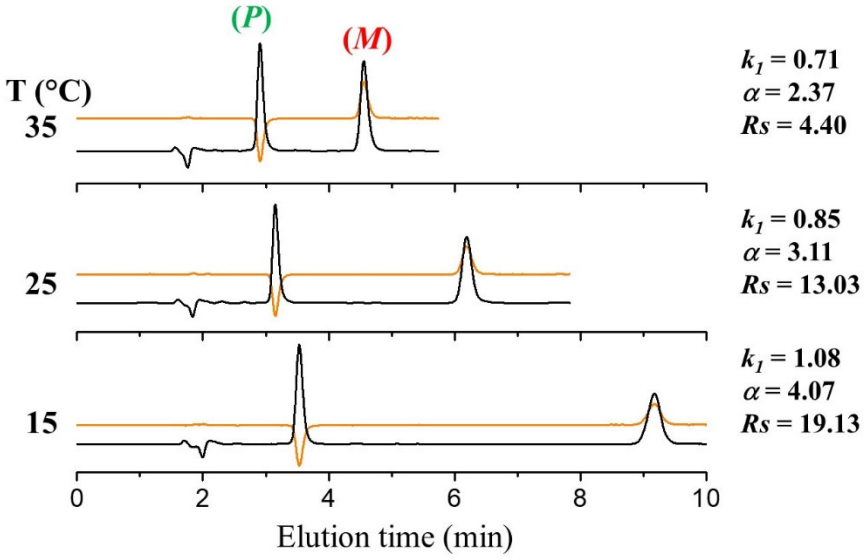

Figure 2. Chromatograms illustrating the effect of column temperature on retention, enantioseparation, and resolution. Chromatographic conditions: column, Chiralpak IB-3 (100 × 4.6 mm, 3 µm); mobile phase, *n*-hexane–methanol–dichloromethane 90:5:5 (*v*/*v*/*v*); column temperature, as indicated in figure; flow rate, 1.0 mL min^{-1}; detection, UV (black) and CD (orange) at 380 nm.

However, to develop an effective semipreparative enantioseparation, the choice of an appropriate diluent is another key step that needs to be considered. In fact, if the solubility of the racemate in the mobile phase is low, the quantity of chiral sample loaded in a single chromatographic run is limited. In the case of coated-type polysaccharide-derived CSPs, which are substantially stable only when n-hexane, alcohols, and their mixtures are used as mobile phases, the productivity of the HPLC resolution of analytes soluble in a chlorinated solvent is low. This problem can be solved using a CSP in which the polymeric selector is immobilized onto the silica matrix, as is in the case of the Chiralpak IB-3 CSP. To evaluate the nature of the diluent to be used to dissolve the racemic **BT2–T6,** their solubility in various solvents was explored. The outcomes of this study demonstrated a low solubility (<1 mg mL^{-1}) of the chiral compound in n-hexane–alcohol mixtures and a very high solubility (>20 mg mL^{-1}) in dichloromethane. Therefore, to accomplish the resolution of **BT2–T6** on a semipreparative scale, dichloromethane was selected as the diluent. This solvent is particularly suitable for applications on the milligram scale, not only for its high solvation power, but also for the high degree of enantioselectivity produced when utilized as an organic solvent in the mobile phase and the ease of removing it from the eluate by evaporation.

The performance of the semipreparative 250 mm × 10 mm Chiralpak IB column was studied using a solution obtained by dissolving 50 mg of racematic racemate **BT2–T6** in 7.5 mL of dichloromethane. During loading studies, the profile of the enantiomeric peaks in the chromatogram was used as a diagnostic to assess the complete separation.

It was observed that just by increasing the racemic sample load from 0.07 mg to 0.67 mg (the injection volumes were 10 and 100 µL), each enantiomeric peak was split into two sub-peaks of similar area (Figure 3).

As the injection volume continued to increase, the elution profile became more distorted and other peaks appeared in the chromatogram. When the injection volume was 500 µL, corresponding to 3.34 mg of *rac*-**BT2–T6** (Figure 4) besides the peak of the enantiomers (*P*) and (*M*), three additional peaks linked to the those of the enantiomers by plateaus were clearly visible. In particular, the peak relevant to the least-retained species

was eluted in the void time and it was composed of an enantioenriched mixture of the enantiomers (*P*) and (*M*) in a 60/40 ratio.

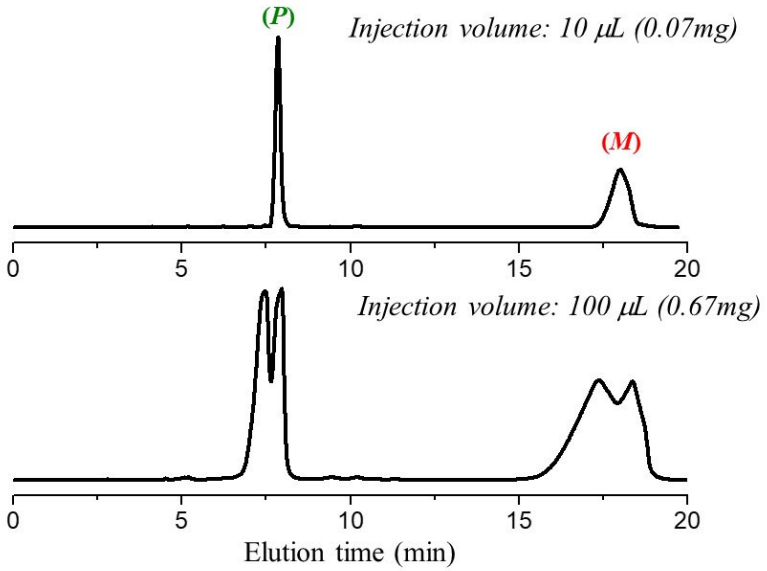

Figure 3. Elution profiles of **BT2–T6** for different sample loads. Chromatographic conditions: column, Chiralpak IB (250 × 10 mm, 5 μm); mobile phase, *n*-hexane–methanol–dichloromethane 90:5:5 (*v*/*v*/*v*); column temperature, 15 °C; flow rate, 5.5 mL min^{-1}; detection, UV at 360 nm.

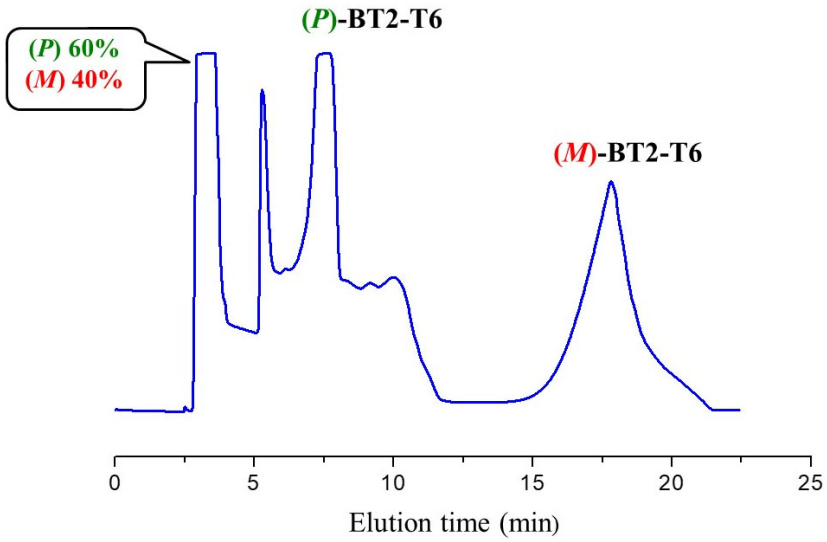

Figure 4. Aberration on the HPLC chromatogram after injection of 3.34 mg of *rac*-**BT2–T6** in 500 μL. of dichloromethane. Chromatographic conditions: column, Chiralpak IB (250 × 10 mm, 5 μm); mobile phase, *n*-hexane–methanol–dichloromethane 90:5:5 (*v*/*v*/*v*); column temperature, 15 °C; flow rate, 5.5 mL min^{-1}; detection, UV at 360 nm.

Thus, it is clear that, although the chromatographic support is able to resolve the enantiomers of **BT2–T6**, such a desired outcome will be hindered by the occurrence of the competitive phenomena.

A possible explanation for the presence of distorted peaks in Figures 3 and 4 could be sample overloading on the column. However, it is useful to remember that whereas an analytical column becomes easily overloaded because of the reduced available active sites, semipreparative columns packed with polysaccharide-based CSPs have a very high sample throughput capability. Many research groups have already reported, in numerous

publications, the resolution of milligram amounts of the racemic compound and injection volumes of 1–5 mL without observing peak overlapping or peak splitting [11,22,23].

Another critical aspect that should be considered to figure out the aberration of the semipreparative chromatogram is the composition of the sample diluent. In particular, when the elution power of the diluent is stronger than that of the mobile phase, undesirable broadening of the bandwidth can occur along the column.

The mismatch of elution strength between the solvents used as the diluent and mobile phase falls in our case because the sample diluent is neat dichloromethane and the mobile phase is n-hexane/methanol/dichloromethane 90:5:5 (*v*/*v*/*v*). When a large amount of sample dissolved in the strong solvent, dichloromethane, is injected into the column running a weak mobile phase, the sample could precipitate immediately and redissolve during the run and cause peak splitting and distortion. In addition, as a result of the mismatch of elution strength, the sample may be weakly retained in the stationary phase, resulting in unretained elution of part of it. The explanation for the anomalous retentive behavior has physical reasons: the analyte has to be solvated in the mobile phase, but its solubility is lower. As a result, a large amount of sampling solvent perturbs the chiral stationary phase and a portion of the analyte is forced to stay in dichloromethane and, consequently, to be eluted with dichloromethane as an unretained peak.

To overcome the effects due to sample–solvent mismatch, it is advisable to dissolve and inject the sample in the same solvents used for the mobile phase. Unfortunately, this strategy can be rarely adopted because of the low solubility of the sample in the mobile phase.

Pure dichloromethane was initially used to dissolve **BT2–T6** because of dichloromethane's excellent solubility. In opposition to dichloromethane, n-hexane is a bad solvent for the compound. The mobile phase in use for chromatography was a mixture of n-hexane/MeOH/dichloromethane 90:5:5 (*v*/*v*/*v*) in which the "bad solvent" is in the large majority.

The way to solve that problem consists of diluting the chlorinated solution with *n*-hexane. However, one should be aware that as the *n*-hexane volume increases, the solubility of the chiral analyte reduces. Thus, different samples containing the same concentration of **BT2–T6,** but increasing percentages of n-hexane were tested. As indicated in Figure 5, good results were obtained by dissolving 5.2 mg of **BT2–T6** in 0.75 mL of dichloromethane and adding 4.0 mL of *n*-hexane to the solution. Under these conditions, the peak splitting was hampered and two well-separated and symmetric enantiomeric peaks were obtained. The enantiomeric forms of **BT2–T6** were collected with high enantiomeric excess (ee > 99%) and yields (95%). Considering that the chromatographic resolution was completed in about 15 min, 10 mg of each enantiomer per hour could be isolated.

3.3. Absolute Configuration Assignment

The availability of pure enantiomers of **BT2–T6** on a multimilligram scale is mandatory to produce homochiral oligomeric enantioselective sensors to apply in electrochemical enantiorecognition studies [3,12–17]. In this type of chiral conducting oligomers, the conjugated system is responsible both for the electrochemical properties and molecular chirality, hence the name inherently chiral. The structural design of the oligomers requires the stereochemical characterization of the enantiopure monomer precursors.

The absolute configuration of enantiomers of **BT2–T6** was empirically established according to their ECD properties. As shown in Figure 6, the first eluted enantiomer, at a concentration of 0.3 mg mL^{-1}, showed a strong positive Cotton effect in dichloromethane solution, with positive and negative ECD bands centered at 433 and 383 nm, respectively. A very similar ECD profile was recorded for the (*P*)-enantiomer of the chiral analog **BT2–T4**, which differs in the number of thiophenes as wings (i.e., four instead of six) and for which the stereochemical characteristics were determined in a preceding work [3]. Thus, the strict correlation between ECD spectra allowed assigning the (*P*) configuration to the first eluted enantiomer of **BT2–T6** on the Chiralpak IB CSP and (*M*) configuration to the more retained enantiomer. Notably, the atropisomeric compounds **BT2–T4** and **BT2–T6** exhibited the

same order of enantiomer elution on the Chiralpak IB CSP with the (*M*)-enantiomer eluted first and the (*P*)-enantiomer eluted second. In addition, it is worth highlighting that the univocal correlation between the CD sign exhibited by the enantiomeric peak (Figure 2) at the wavelength of 380 nm and absolute configuration (i.e., the negative CD sign corresponds to (*P*) absolute configuration and vice versa). This means that the online CD detection during HPLC enantioseparation provides a direct readout of the stereochemistry of **BT2–T4** and **BT2–T6**.

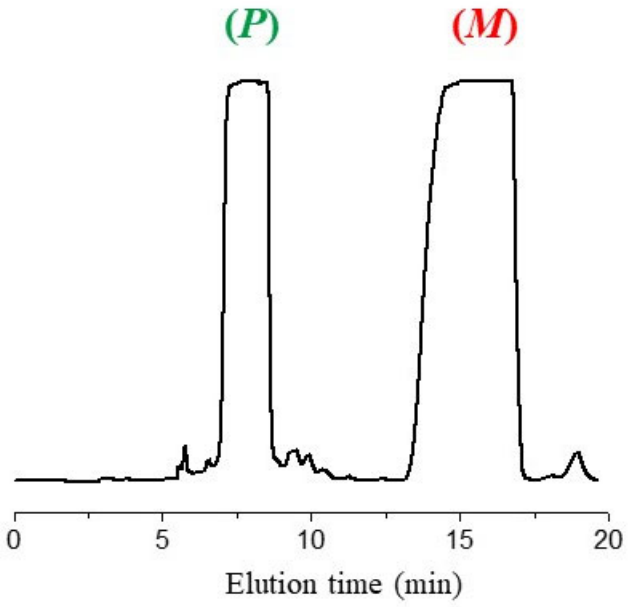

Figure 5. Optimized semipreparative HPLC resolution of 5.2 mg of **BT2–T6**. Chromatographic conditions: column, Chiralpak IB (250 mm × 10 mm, 5 µm); mobile phase, *n*-hexane–methanol–dichloromethane 90:5:5 (*v*/*v*/*v*); column temperature, 15 °C; flow rate, 5.5 mL min^{-1}; detection, UV at 360 nm.

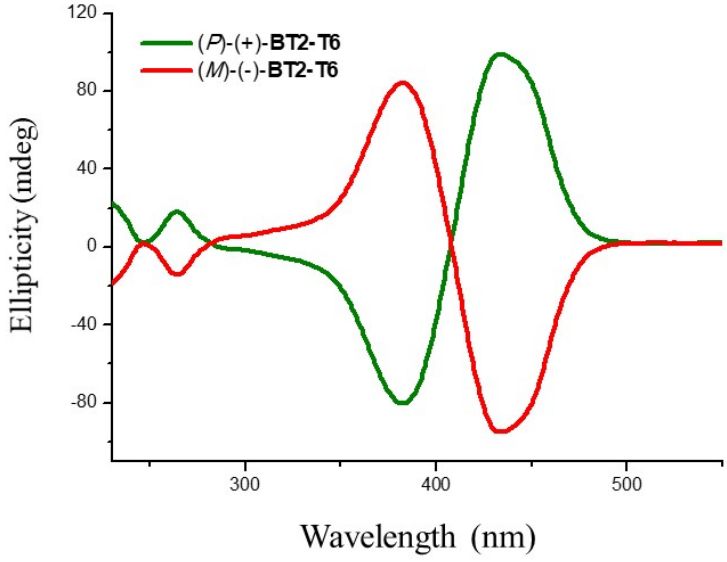

Figure 6. ECD spectra of the enantiomers of **BT2–T6**.

As for **BT2–T4** case, the (*P*)-enantiomer was dextrorotatory and the value of the specific rotation at a wavelength of 589 nm was remarkable, at +1821 (c = 0.4, dichloromethane).

4. Conclusions

A further member of C_2-symmetry atropisomeric thiophene-based monomers for the production of inherently chiral electroactive materials has been synthesized and its chiroptical and stereochemical properties fully characterized.

It was demonstrated that the optimization of the chromatographic parameters that control the enantioseparation process of **BT2–T6** on the Chiralpak IB CSP, namely column temperature and mobile phase composition, as well as the appropriate choice of nature of diluent, can yield high-throughput semipreparative conditions.

The wide availability of the enantiomers of the thiophene-based monomer contributes to: (i) exploring the applicability of the pertinent inherently chiral oligomer materials in chiral bipolar electrochemistry for the development of on–off sensors capable of quantifying the enantiomeric purity of enantioenriched chiral probes, including chiral active pharmaceutical ingredients [16]; (ii) establishing the influence of more extensive wings of the atropisomeric scaffold of the chiral molecule on the oxidative electrodeposition of the electroactive film and to evaluate its enantioseparation capability for different chiral probes and operating conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13031407/s1, Figure S1. ^{1}H NMR and ^{13}C NMR spectra of **BT2-T6** recorded in $CDCl_3$ on a 300 MHz spectrometer.

Author Contributions: A.R.: performed the HPLC and ECD experiments. G.A.: synthesized the **BT2–T6** molecule. C.V.: discussed the results and edited the manuscript. T.B.: designed the chiral **BT2–T6** molecule, and wrote and edited the manuscript. R.C.: proposed and supervised the research project, designed the experiments, performed the HPLC and ECD experiments, and wrote and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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