Spin-labeled Diclofenac: Synthesis and Interaction with Lipid Membranes

Denis S. Baranov , Anna S. Smorygina , Anastasiya N. Atnyukova , Sergey A Dzuba *

Posted Date: 31 July 2023

doi: 10.20944/preprints202307.2088.v1

Keywords: non-steroidal anti-inflammatory drug; NSAID; lipid bilayer; EPR; 2H ESEEM

Article

Spin-Labeled Diclofenac: Synthesis and Interaction with Lipid Membranes

Denis S. Baranov [1], Anna S. Kashnik [1], Anastasiya N. Atnyukova [2] and Sergei A. Dzuba [1,*]

[1] Voevodsky Institute of Chemical Kinetics and Combustion, SB RAS, 630090 Novosibirsk, Russian Federation
[2] Department of Physics, Novosibirsk State University, 630090 Novosibirsk, Russian Federation
* Correspondence: dzuba@kinetics.nsc.ru (S.A.D.)

Abstract: Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) from the group of phenylacetic acid derivatives, which has analgesic, anti-inflammatory and antipyretic properties. The interaction of non-steroidal anti-inflammatory drugs with cell membranes can affect their physicochemical properties, which, in turn, can cause a number of side effects in the use of these drugs. Electron paramagnetic resonance (EPR) spectroscopy could be used to study the interaction of diclofenac with the membrane, if its spin-labeled analogs existed. This paper describes the synthesis of spin-labeled diclofenac (diclofenac-SL), which consists of a simple sequence of transformations such as iodination, esterification, Sonogashira cross-coupling, oxidation and saponification. EPR spectra showed that diclofenac-SL binds to in a lipid membrane composed of palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). ^{2}H electron spin echo spectroscopy (ESEEM) was used to determine the position of the diclofenac-SL relative to the membrane surface. It has been established that the average its depth of immersion corresponds to the 5th position of the carbon atom in the lipid chain.

Keywords: non-steroidal anti-inflammatory drug; NSAID; lipid bilayer; EPR; ^{2}H ESEEM

1. Introduction

Non-steroidal anti-inflammatory drugs are effective antipyretic and analgesic pharmacological agents [1], which are also used in the treatment of other diseases, including cancer [2], arthritis [3,4] and neurodegenerative diseases [5]. The most commonly used NSAIDs are ibuprofen, naproxen, and diclofenac [6]. Due to their wide application, these compounds are found even in the environment in the form of human waste [7].

Like all NSAIDs, diclofenac works by inhibiting the activity of the enzyme cyclooxygenase (COX) to disrupt prostaglandin synthesis [8]. However, alternative mechanisms of action of NSAIDs associated with the membrane activity of drugs are known [9–11]. For example, changes in lipid composition and/or membrane structure can lead to the development of various cardiovascular pathologies, including hypertension, myocardial infarction and thrombosis [12]. Diclofenac has demonstrated the ability to chemically interact with gastrointestinal protective barrier phospholipids, which may contribute to its gastrointestinal toxicity [13,14]. Therefore, understanding the mechanisms of interaction of diclofenac with the plasma membrane at the molecular level can be extremely useful for understanding the therapeutic effect of this drugs and developing ways to limit the side effects.

Among the huge variety of experimental approaches aimed at studying the molecular mechanisms of the interaction of NSAIDs with membranes [6,9–11,13–23], one can also use electron paramagnetic resonance (EPR) spectroscopy of spin-labeled drug molecules [24]. Spin-labeled drugs allow the study of intermolecular interactions and the use of low concentrations that can be close to therapeutic doses. This paper describes the synthesis of spin-labeled diclofenac and its interaction with a model membrane characterized by conventional and pulsed EPR.

To introduce a nitroxide spin label into drugs that are derivatives of carboxylic acids, the use of transformations of carboxyl groups is the simplest way. Different variants of esterification and amidation reactions are well developed and widely used for the synthesis of esters and amides of many drugs [25–28]. For example, spin-labeled esters and amides of aspirin [29], ibuprofen [30],

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 July 2023 doi:10.20944/preprints202307.2088.v1

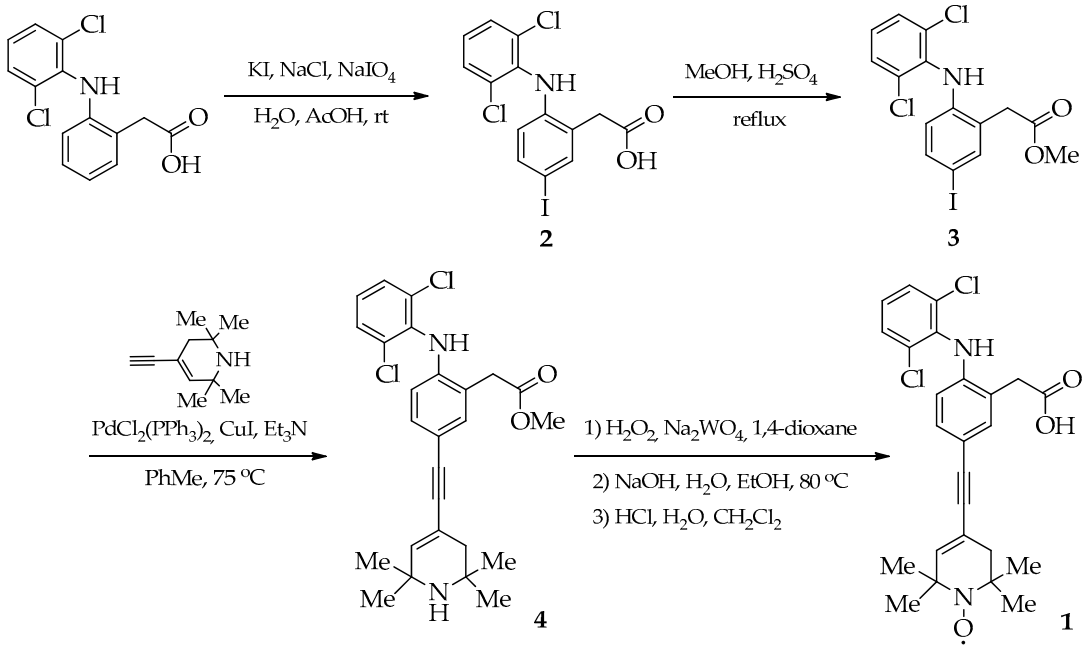
indomethacin [31] and diclofenac [32,33] were synthesized for their study by EPR. However, the loss of the carboxyl group in the original molecule of drugs is a significant drawback for their use as model objects of study. Indeed, the data from studies of the processes of action of non-steroidal anti-inflammatory drugs [34], as well as the fact that the vast majority of them are derivatives of organic acids, convincingly indicate the exceptional importance of the carboxyl group.

Recently, we have developed an alternative route to introduce the nitroxyl label into ibuprofen via ethynylation of the benzene ring while retaining the carboxyl group and its associated molecular properties, in particular amphiphilicity [24]. Here we report procedures for the synthesis of a novel diclofenac derivative in which the nitroxyl radical is linked to the benzene ring through an alkyne moiety distant from the carboxymethyl group.

2. Results and Discussion

2.1. Synthesis

The sequence of chemical transformation carried out in this work is shown in Scheme 1. To obtain spin-labeled diclofenac **1** (diclofenac-SL), we used a four-step synthesis, which is a new and simpler modification of our approach previously developed for ibuprofen [24]. Initially, iododiclofenac **2** was obtained in 89% yield by the oxidative iodination of diclofenac with KI, $NaIO_4$ and NaCl in acetic acid at room temperature. Then, iododiclofenac was converted into its methyl ester **3** in 95% yield by an esterification reaction in boiling methanol with sulfuric acid. In the next step, Pd/Cu-catalyzed Sonogashira coupling reaction of iodide with 4-ethynyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridine in the presence of Et_3N at 75 °C gave the precursor **4** (87% yield). Finally, the precursor was subjected to peroxidation in dioxane followed by saponification with aqueous sodium hydroxide to the target spin-labeled product **1** in 34% yield.



Scheme 1. Synthesis of diclofenac-SL **1**.

Diclofenac has a tendency to complex degradation when interacting with hydrogen peroxide in the presence of catalysts, such as peroxidases [35,36]. The poor yield of the key product in the last step indicates that the Na_2WO_4 acts similarly to peroxidases. In addition, there is a gradual reduction of the compound during purification and storage, which is typical of many nitroxide radicals.

The structure and purity of the synthesized compounds **1**-**4** were confirmed by ^{1}H-NMR and ^{13}C-NMR (see Supplementary Materials). According to the ^{1}H-NMR spectra of the product, after a month of storage in the environment (see Figure S7), an accumulation of impurities in the region of 1.3 ppm (Me groups) was observed.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 July 2023 doi:10.20944/preprints202307.2088.v1

2.2. EPR Spectra: Interaction with the POPC Membrane

The CW EPR spectra at room temperature (25 °C) obtained for diclofenac-SL are shown in Figure 1. In Figure 1a the spectrum is given for diclofenac-SL dissolved in toluene at a concentration of 5 mM. This spectrum is characteristic of nitroxide in an isotropic medium in a state of fast tumbling motion of a spin label in solution [37]. The value of the *g*-factor for this spectrum is 2.0023 ± 0.0001 and the hyperfine interaction constant a = 1.5 ± 0.02 mT.

Figure 1b shows the EPR spectra for diclofenac-SL in palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) multilamellar vesicles. Samples were prepared in two ways (see Materials and Methods below). In the first one, vesicles were prepared from the initial mixture of diclofenac-SL/POPC mixture (1:99 mol/mol). In the second, diclofenac dissolved in dimethyl sulfoxide (DMSO) was added to pure POPC vesicles. One can see from Figure 1b that the difference in the EPR spectra for two different preparation methods is insignificant.

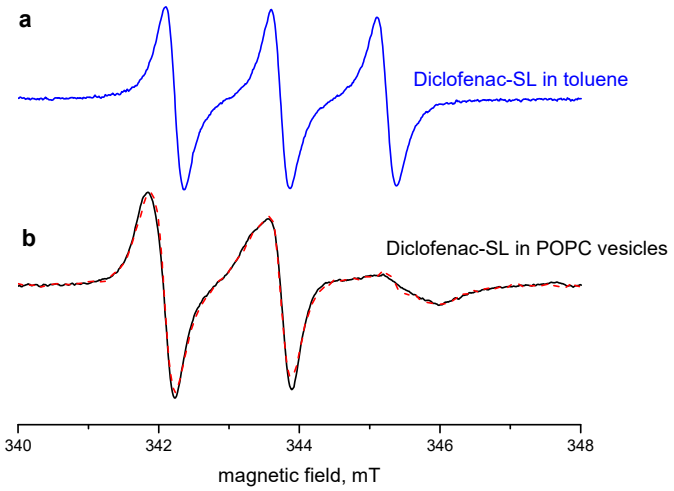


Figure 1. EPR spectra at room temperature of diclofenac-SL for its 5 mM solution in toluene (a) and in the presence of POPC vesicles at its concentration of 1 mol% (b). In the latter case, the samples were prepared either by mixing diclofenac-SL and POPC prior the sample preparation (solid line), or by adding the solution of diclofenac-SL in DMSO to the prepared vesicles (dashed line).

It is important that the spectra in Figure 1b show a significant decrease in the high-field component compared to other components (cf. Figure 1a). This decrease indicates a substantial retardation of the motion [37] which can take place only if the molecules are bound to the membrane. Thus, these data clearly indicate that diclofenac-SL is incorporated into the membrane.

2.3. Pulsed EPR: Location in the POPC Membrane

^{2}H ESEEM spectroscopy is capable of providing information about the spatial position of the spin label [38–40]. The ESEEM effect is caused by an anisotropic hyperfine interaction of an unpaired electron of a spin label electron with neighboring nuclei at distances of less than 1 nm [38–40]. If the membrane is hydrated with deuterium water, then ^{2}H ESEEM makes it possible to determine the position of the spin label relative to the membrane surface.

The ^{2}H ESEEM time traces for diclofenac-SL in D_2O-hydrated POPC bilayers were refined from the background spin relaxation by dividing by the mean echo decay, as described previously [38–40]. Figure 2a shows these refined ESEEM time traces for diclofenac-SL in a D_2O-hydrated POPC vesicles. Figure 2a also provides reference data on stearic acids, doxyl-spin-labeled at *n*-th carbon atom positions along the carbon chain, *n*-DSA [24], and data [41] on 2-oleoyl-1-palmitoyl-sn-glycero-3-phospho(tempo)choline (TEMPO-PC). In TEMPO-PC the spin label is attached to the polar lipid head. The corresponding Fourier transforms are shown in Figure 2b.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 July 2023 doi:10.20944/preprints202307.2088.v1

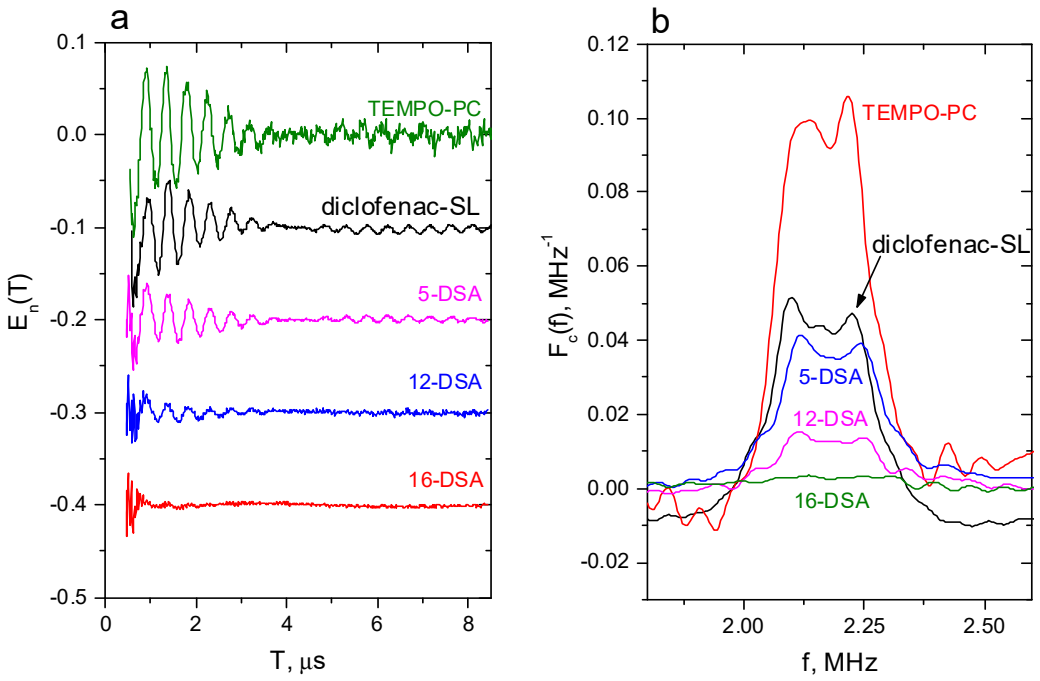


Figure 2. (a) ESEEM time traces for diclofenac-SL in D_2O-hydrated POPC bilayer. For comparison, the analogous data for TEMPO-PC and 5(12,16)-DSA (see text) are given. The data are vertically shifted for convenience. (b) Their Fourier transforms.

It can be seen that the TEMPO-PC sample shows the highest amplitude, which is obviously due to the fact that the spin label is located directly in the water shell. For spin-labeled stearic acids, *n*-DSA, the signal amplitude becomes smaller with increasing *n*, which corresponds to immersion inside the membrane. For diclofenac-SL, the ESEEM amplitude shows close proximity to that of the 5-DSA sample. So, we may conclude that the spin label is embedded into the membrane interior and that its mean position corresponds to the 5th position of the carbon atom in the lipid chain.

3. Materials and Methods

3.1. Chemical Analysis

^1H and ^{13}C spectra were recorded with Bruker AV-500 (500 (^1H) and 126 (^{13}C) MHz) spectrometer in $CDCl_3$ or DMSO-D_6 solvents, by using residual signals of undeuterated solvents ($CHCl_3$: δ= 7.26 ppm for ^1H and δ = 77.16 for ^{13}C ppm. DMSO: δ= 2.50 ppm for ^1H) as an internal standard. Melting points were measured with Electrothermal MEL-TEMP 1101D apparatus. HRMS was recorded with a Thermo Scientific DFS high-resolution mass spectrometer (Thermo Electron Corp., USA). The IR spectra were obtained on a Shimadzu IRTracer-100 instrument with GS10802-X Quest ATR ZnSe Accessory (Specac). Column chromatography was carried out using silica gel (70−230 mesh ASTM). All reagents and solvents were obtained from commercial sources and used without special purification. Diclofenac was purchased from Sigma-Aldrich.

3.2. Synthesis and Characterization

4-Ethynyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridine was obtained according to the method reported in [42].

{2-[(2,6-Dichlorophenyl)amino]-5-iodophenyl}acetic acid (**2**). KI (1.8 g, 10.8 mmol) was added in several portions to a stirred mixture of diclofenac (3.2 g, 10.8 mmol), NaCl (1.26 g, 21.5 mmol), $NaIO_4$ (2.3 g, 10.8 mmol) and H_2O (10 mL) in AcOH (60 mL) at room temperature for 24 h. After the disappearance of the iodine color, the precipitate was filtered, washed with H_2O and dried in air. The crude product was purified by flash chromatography on silica gel (eluent: ethyl acetate). Yield 4.0 g (88%), white solid, mp 191-193 °C (toluene). ^1H-NMR (500 MHz, DMSO-D_6) δ: 7.53 (m, 3H), 7.35 (d, J

 doi:10.20944/preprints202307.2088.v1

= 8.4 Hz, 1H), 7.31 (s, 1H), 7.23 (m, 1H), 6.04 (d, J = 8.4 Hz, 1H), 3.68 (s, 2H) (see ^{1}H-NMR in [43,44]). IR (film) cm^{-1}: 3352 (NH), 3182, 3028, 2814 (OH), 1693 (C=O).

Methyl {2-[(2,6-dichlorophenyl)amino]-5-iodophenyl}acetate (**3**). A mixture of acid **2** (2.0 g, 4.75 mmol) and concentrated H_2SO_4 (1 mL) in methanol (45 mL) was stirred at reflux for 0.3 h. Then, the reaction mixture was cooled to room temperature; the precipitate was isolated by filtration, washed with methanol and dried in air. Yield 1.96 g (95%), white solid, mp 119-120 °C (methanol). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.53 (d, J = 1.5 Hz, 1H), 7.39 (dd, J = 8.5, 1.8 Hz, 1H), 7.35 (d, J = 8.0 Hz, 2H), 7.01 (t, J = 8.0 Hz, 1H), 6.94 (br.s, 1H), 6.28 (d, J = 8.5 Hz, 1H), 3.76 (s, 3H), 3.75 (s, 2H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ: 172.24, 142.83, 139.44, 137.19, 136.95, 129.96, 129.08, 126.29, 124.78, 119.97, 84.13, 52.73, 38.16. IR (film) cm^{-1}: 3321 (NH), 2949 (Me), 1714 (C=O). HRMS (ESI) m/z: $[M]^+$ Calcd for $C_{15}H_{12}Cl_2INO_2$ 434.9284. Found 434.9285.

Methyl {2-[(2,6-dichlorophenyl)amino]-5-[(2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridin-4-yl)ethynyl]phenyl}acetate (**4**). Under argon atmosphere, a mixture of iodide **3** (500 mg, 1.15 mmol), $PdCl_2(PPh_3)_2$ (20 mg, 0.03 mmol) and CuI (10 mg, 0.05 mmol) in toluene (20 mL) was stirred and heated to 55 °C. Then, Et_3N (8 mL) and 4-ethynyl-2,2,6,6-tetramethyl-1,2,3,6-tetrahydropyridine (270 mg, 1.65 mmol) was added, and the reaction mixture was stirred at 75 °C for 16 h. After cooling, toluene (100 mL) was added and the mixture was filtered. Extract was washed with 6 M aqueous ammonia (2 × 200 mL) and dried over $MgSO_4$. The crude product was purified by column chromatography on silica gel (eluent: toluene / ethyl acetate, 4:1). Yield 470 mg (87%), white solid, mp 150-151 °C (ethyl acetate). ^{1}H-NMR (500 MHz, $CDCl_3$) δ : 7.34 (m, 3H, H_{Ar}), 7.19 (m, 1H), 7.11 (s, 1H), 7.01 (t, J = 8.0 Hz, 1H), 6.45 (d, J = 8.2 Hz, 1H), 6.05 (s, 1H), 3.76 (s, 2H), 3.75 (s, 3H), 2.87 (br.s, 1H), 2.07 (s, 2H), 1.25 (s, 6H), 1.20 (s, 6H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ: 172.52, 142.71, 140.23, 137.06, 134.25, 131.34, 129.90, 129.04, 124.68, 123.59, 117.68, 116.46, 116.33, 89.61, 87.68, 52.70, 51.91, 49.71, 41.03, 38.46, 30.94, 29.75. IR (film) cm^{-1}: 3348 (NH), 2961 (Me), 1745 (C=O). HRMS (ESI) m/z: $[M]^+$ Calcd for $C_{26}H_{28}Cl_2N_2O_2$ 470.1522. Found 470.1521.

[4-{[3-(Carboxymethyl)-4-((2,6-dichlorophenyl)amino)phenyl]ethynyl}-2,2,6,6-tetramethyl-3,6-dihydropyridin-1(2H)-yl]oxidanyl (**1**). A mixture of compound **4** (120 mg, 0.25 mmol), $Na_2WO_4{\cdot}2H_2O$ (13 mg, 0.04 mmol), EDTA disodium salt (13 mg, 0.04 mmol) and 30% H_2O_2 (0.3 mL) in 1,4-dioxane (3 mL) was stirred at ambient temperature for 14 days. The suspension was filtered, the solvent was evaporated under vacuum condition. Then, 2 M NaOH (3 mL) was added, and the reaction mixture was stirred at 80 °C for 0.5 h. Next, the resulting mixture was diluted with ethanol (3 mL) and stirred at reflux for 0.25 h. After cooling, H_2O (5 mL) and CH_2Cl_2 (15 mL) were added. At 0 °C, 1 M HCl was added dropwise to the stirred mixture until pH was neutral. The organic layer was separated and dried over $MgSO_4$. The solvent was evaporated under vacuum condition. The crude product was purified by column chromatography on silica gel (eluent: toluene / ethyl acetate, 5:1). Yield 40 mg (34%), white solid, mp 190-192 °C (toluene). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 7.51 (m, 4H), 7.10 (s, 1H), 6.95 (s, 1H), 6.46 (s, 1H), 3.98 (s, 2H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ: 176.065, 140.80, 135.85, 130.19, 128.73, 128.02, 127.33, 123.77, 122.90, 119.32, 117.51, 38.66. IR (film) cm^{-1}: 3300 (NH), 3042 (OH), 2928, 2851 (Me), 2203 (C≡C), 1695 (C=O). HRMS (ESI) m/z: $[M]^+$ Calcd for $C_{25}H_{25}Cl_2N_2O_3$ 471.1237. Found 471.1235.

3.3. Sample Preparations for EPR Investigation

For obtaining EPR spectra in toluene solution, diclofenac-SL **1** was dissolved in toluene (Ekros-Analytica, St. Petersburg, Russia, distilled before the use) at concentration of 5 mM. Lipids POPC were from Avanti Polar Lipids (Birmingham, AL, USA).

The vesicle samples were prepared in two ways. First, POPC and diclofenac-SL were dissolved separately in chloroform, then two solutions were mixed, so that diclofenac-SL/POPC molar ratio was 1:99. The solvent was removed in the nitrogen stream, with subsequent storing of the mixture under vacuum for 4 h. Then the phosphate-buffered saline (pH 7.0) was added to the resulting powder in a proportion of 10:1. The sample was stirred, then stored for 2 h and the resulted vesicles were centrifuged to remove the excess solvent. Instead of ordinary water, deuterium-substituted water was used in some measurements.

For the second way of sample preparation, POPC vesicles were prepared in the same way but without diclofenac-SL, with the subsequent addition of dimethylsulfoxide (DMSO) solution of

diclofenac-SL (DMSO content was less than 10 vol % respectively to the sample volume). The total diclofenac-SL mole content was 100 times smaller than the total lipid mole content.

The samples were placed in glass EPR tubes with an outer diameter of 3 mm. and examined either at room temperature (25°C) or at 80 K. In the latter case, the samples were quickly frozen by immersion in liquid nitrogen.

3.4. EPR Measurements

Conventional EPR spectra were obtained at room temperature with a Bruker ESP 380E spectrometer operating at modulation amplitude of 0.01 mT, with the output microwave (MW) power of 100 mW, the MW attenuation set up to 25 dB, the sweep and constant times of 60 s and 46 ms, respectively. Bruker ER 4118 X-MD-5 dielectric resonator was used. In pulsed EPR studies, an X-band Bruker ELEXSYS E580 EPR spectrometer was used equipped with a split-ring Bruker ER 4118 X-MS-3 resonator and an Oxford Instruments CF-935 cryostat.

The three-pulse ESEEM sequence $(\pi/2)$-τ-$(\pi/2)$-t-$(\pi/2)$-τ-echo was employed, with excitation at the maximum of the echo-detected EPR spectrum. The pulse lengths were 16 ns, time delay τ was 204 ns, the time delay t was scanned from 300 ns to 10 μs, with a 12 or 16 ns time step. The resonator was cooled with a stream of cold nitrogen gas. The temperature was controlled by a nitrogen flow stabilized by a Bruker ER4131VT temperature controller. The sample temperature was kept near 80 K.

4. Conclusions

This work shows that the NSAID diclofenac can be spin-labeled through a simple four-step sequence that includes iodination, esterification, Sonogashira cross-coupling, oxidation, and saponification. The obtained CW EPR data indicate that diclofenac-SL binds to model lipid membranes. The ESEEM spectroscopy shows that diclofenac molecules are located under the polar heads of lipids with an average position near the 5th position of the carbon of the lipid chain.

Note that a standard problem with the labeling approach is that the label distorts the original molecule. In our case, the main structural properties of the diclofenac molecule are a polar carboxyl residue and a Cl-containing aromatic ring, and these properties are not violated in the suggested labeling scheme. On the other hand, the advantages of spin-label EPR are that it provides information at the molecular level and allows the use of very low concentrations. In addition, pulsed EPR in the version of double electron-electron resonance (DEER, also known as PELDOR) provides the possibility of obtaining data on nanoscale clustering of mono-spin-labeled molecules [45–48].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/, Figure S1: ^{1}H-NMR spectrum of compound **2** in DMSO-D_6; Figures S2-S6: ^{1}H-NMR spectrum of compounds **3 - 4** in $CDCl_3$; Figure S6: ^{1}H-NMR spectrum of compound **1** in $CDCl_3$; Figure S7: ^{1}H-NMR spectrum of compound **1** in $CDCl_3$ after one month of storage in the environment; Figure S8: ^{13}C-NMR spectrum of compound **1** in $CDCl_3$.

Author Contributions: Methodology, synthesis and writing, D.S.B.; investigation, data curation and writing, A.S.S.; investigation, A.N.A.; conceptualization, writing – review and editing, S.A.D. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Russian Science Foundation, project # 21-13-00025.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the synthesized compounds are available from the authors.

References

1. Manrique-Moreno, M.; Heinbockel, L.; Suwalsky, M.; Garidel, P.; Brandenburg, K. Biophysical study of the non-steroidal anti-inflammatory drugs (NSAID) ibuprofen, naproxen and diclofenac with phosphatidylserine bilayer membranes. *Biochim. Biophys. Acta.* **2016**, 1858, 2123–2131.
2. Ulrich, C.M.; Bigler, J.; Potter, J.D. Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. *Nature Reviews Cancer*. **2006**, 6, 130-140.
3. Crofford, L.J. Use of NSAIDs in treating patients with arthritis. *Arthritis Res. Ther.* **2013**, 15, S2.
4. González-Gay, M.A.; González-Juanatey. C. NSAIDs and cardiovascular risk in arthritis. *Nat. Rev. Cardiol*. **2017**, 14, 69-70.
5. Lleo, A., Galea, E. & Sastre, M. Molecular targets of non-steroidal anti-inflammatory drugs in neurodegenerative diseases. Cell. Mol. Life Sci. **2007**, 64, 1403–1418. https://doi.org/10.1007/s00018-007-6516-1.
6. Moreno, M.M.; Garidel, P.; Suwalsky, M.; Howe, J.; Brandenburg, K. The membrane-activity of ibuprofen, diclofenac, and naproxen: a physico-chemical study with lecithin phospholipids. Biochim. Biophys. Acta Biomembr. **2009**, 1788, 1296–1303.
7. Madikizela, L.M.; Chimuka, L. Determination of ibuprofen, naproxen and diclofenac in aqueous samples using a multi-template molecularly imprinted polymer as selective adsorbent for solid-phase extraction, J. Pharm. Biomed. Analysis, 2016, 128, 210-215, https://doi.org/10.1016/j.jpba.2016.05.037.
8. Warner, T. D., Giuliano, F., Vojnovic, I., Bukasa, A., Mitchell, J. A. and Vane, J. R., Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis. *Proc. Natl. Acad. Sci.* **1999**, 96, 7563–7568.
9. Leite, C.P.; Nunes, C.; Reis. S. Interaction of nonsteroidal anti-inflammatory drugs with membranes: in vitro assessment and relevance for their biological actions. *Prog. Lipid Res*. **2013**, 52, 571-584.
10. Zhou, Y.; Plowman, S.J.; Lichtenberger, L.M.; Hancock, J.F. The anti-inflammatory drug indomethacin alters Nanoclustering in synthetic and cell plasma membranes. *J. Biol. Chem.* **2010**, 285, 35188-35195.
11. Seddon, A.M.; Casey, D.; Law, R.V.; Gee, A.; Templer, R.H.; Ces, O. Drug interactions with lipid membranes. *Chem. Soc. Rev*. **2009**, 38, 2509-2519.
12. Wallace, JL. Prostaglandins, NSAIDs, and gastric mucosal protection: why doesn't the stomach digest itself? *Physiol Rev.* **2008**, 88, 1547–65.
13. Fernandes, E; Soares, T.B.; Gonçalves, H.; Bernstorff S.; Real Oliveira MECD; Lopes C.M., Lúcio, M. A Molecular Biophysical Approach to Diclofenac Topical Gastrointestinal Damage. *Int J Mol Sci.* **2018**, 19, 3411.
14. Lichtenberger, L. M.; Zhou, Y.; Dial, E. J., Raphael, R.M. NSAID injury to the gastrointestinal tract: evidence that NSAIDs interact with phospholipids to weaken the hydrophobic surface barrier and induce the formation of unstable pores in membranes. *J Pharm Pharmacol.* **2006**, 58, 1421-8.
15. Alsop, R.J.; Armstrong, C.L.; Maqbool, A.; Toppozini, L.; Dies, H.; Rheinstädter, M.C. Cholesterol expels ibuprofen from the hydrophobic membrane core and stabilizes lamellar phases in lipid membranes containing ibuprofen. Soft Matter **2015**, 11, 4756–4767.
16. Sreij, R.; Prévost, S.; Dargel, C.; Dattani, R.; Hertle, Y.; Wrede, O.; Hellweg, T. Interaction of the Saponin Aescin with Ibuprofen in DMPC Model Membranes. Mol. Pharm. **2018**, 15, 4446–4461.
17. Sharma, V.K.; Mamontov, E.; Tyagi, M. Effects of NSAIDs on the nanoscopic dynamics of lipid membrane. Biochim. Biophys. Acta Biomembr. **2020**, 1862, 183100.
18. Yefimova, S.L.; Tkacheva, T.N.; Kasian, N.A. Study of the Combined Effect of Ibuprofen and Cholesterol on the Microviscosityand Ordering of Model Lipid Membranes by Timeresolved Measurement of Fluorescence Anisotropy Decay. J. Appl. Spectrosc. **2017**, 84, 284–290.
19. Ramadurai, S.; Sarangi, N.K.; Maher, S.; Macconnell, N.; Bond, A.M.; McDaid, D.; Flynn, D.; Keyes, T.E. Microcavity-Supported Lipid Bilayers; Evaluation of Drug-Lipid Membrane Interactions by Electrochemical Impedance and Fluorescence Correlation Spectroscopy. Langmuir **2019**, 35, 8095–8109.
20. Wood, M.; Morales, M.; Miller, E.; Braziel, S.; Giancaspro, J.; Scollan, P.; Rosario, J.; Gayapa, A.; Krmic, M.; Lee, S. Ibuprofen and the Phosphatidylcholine Bilayer: MembraneWater Permeability in the Presence and Absence of Cholesterol. Langmuir **2021**, 37, 4468–4480.
21. Sun, S.; Sendecki, A.M.; Pullanchery, S.; Huang, D.; Yang, T.; Cremer, P.S. Multistep Interactions between Ibuprofen and Lipid Membranes. Langmuir **2018**, 34, 10782–10792.
22. Kremkow, J.; Luck, M.; Huster, D.; Müller, P.; Scheidt, H.A. Membrane interaction of ibuprofen with cholesterol-containing lipid membranes. Biomolecules **2020**, 10, 1384.
23. Aloi, E.; Rizzuti, B.; Guzzi, R.; Bartucci, R. Association of ibuprofen at the polar/apolar interface of lipid membranes. Arch. Biochem. Biophys. **2018**, 654, 77–84.
24. Baranov, D. S.; Smorygina, A. S.; Dzuba, S. A. Synthesis of Spin-Labeled Ibuprofen and Its Interaction with Lipid Membranes. Molecules **2022**, 27 (13), 4127. https://doi.org/10.3390/molecules27134127

25. Kozak, K. R.; Prusakiewicz, J. J.; Rowlinson, S. W.; Marnett, L. J. Enantiospecific, Selective Cyclooxygenase-2 Inhibitors. Bioorganic Med. Chem. Lett. **2002**, 12 (9), 1315–1318. https://doi.org/10.1016/S0960-894X(02)00133-6.
26. Jilani, J.; Idkaidek, N.; Alzoubi, K. Synthesis, In Vitro and In Vivo Evaluation of the N-Ethoxycarbonylmorpholine Ester of Diclofenac as a Prodrug. *Pharm*. **2014**, 7 (4), 453–463. https://doi.org/10.3390/ph7040453.
27. Tammara, V. K.; Narurkar, M. M.; Crider, A. M.; Khan, M. A. Synthesis and Evaluation of Morpholinoalkyl Ester Prodrugs of Indomethacin and Naproxen. *Pharm. Res.* **1993**, 10 (8), 1191–1199. https://doi.org/10.1023/A:1018976520391.
28. Gupta, K.; Kaub, C. J.; Carey, K. N.; Casillas, E. G.; Selinsky, B. S.; Loll, P. J. Manipulation of Kinetic Profiles in 2-Aryl Propionic Acid Cyclooxygenase Inhibitors. *Bioorganic Med. Chem. Lett.* **2004**, 14 (3), 667–671. https://doi.org/10.1016/j.bmcl.2003.11.034.
29. Akdogan, Y.; Emrullahoglu, M.; Tatlidil, D.; Ucuncu, M.; Cakan-Akdogan, G. EPR Studies of Intermolecular Interactions and Competitive Binding of Drugs in a Drug–BSA Binding Model. *Physical Chemistry Chemical Physics*. **2016**, 18 (32), 22531–22539. https://doi.org/10.1039/C6CP04137J.
30. Sasaki, K.; Ito, T.; Fujii, H. G.; Sato, S. Synthesis and Reduction Kinetics of Five Ibuprofen–Nitroxides for Ascorbic Acid and Methyl Radicals. *Chem. Pharm. Bull.* **2016**, 64 (10), 1509–1513. https://doi.org/10.1248/cpb.c16-00347.
31. Flores-Santana, W.; Moody, T.; Chen, W.; Gorczynski, M. J.; Shoman, M. E.; Velázquez, C.; Thetford, A.; Mitchell, J. B.; Cherukuri, M. K.; King, S. B.; Wink, D. A. Nitroxide Derivatives of Non-Steroidal Anti-Inflammatory Drugs Exert Anti-Inflammatory and Superoxide Dismutase Scavenging Properties in A459 Cells. *Br. J. Pharmacol.* **2012**, 165 (4b), 1058–1067. https://doi.org/10.1111/j.1476-5381.2011.01527.x.
32. Golubeva, E. N.; Chumakova, N. A.; Kuzin, S. V.; Grigoriev, I. A.; Kalai, T.; Korotkevich, A. A.; Bogorodsky, S. E.; Krotova, L. I.; Popov, V. K.; Lunin, V. V. Paramagnetic Bioactives Encapsulated in Poly(D,L-Lactide) Microparticules: Spatial Distribution and in Vitro Release Kinetics. *J. Supercrit. Fluids*. **2020**, 158, 104748. https://doi.org/10.1016/j.supflu.2019.104748.
33. Chumakova, N. A.; Golubeva, E. N.; Kuzin, S. V.; Ivanova, T. A.; Grigoriev, I. A.; Kostjuk, S. V.; Melnikov, M. Y. New Insight into the Mechanism of Drug Release from Poly(d,l-Lactide) Film by Electron Paramagnetic Resonance. *Polym. J.* **2020**, 12 (12), 3046. https://doi.org/10.3390/polym12123046.
34. Rainsford, K. D. Ibuprofen: Discovery, Development and Therapeutics; Rainsford, K. D., Ed.; Wiley, 2015. https://doi.org/10.1002/9781118743614.
35. Galati, G.; Tafazoli, S.; Sabzevari, O.; Chan, T. S.; O'Brien, P. J. Idiosyncratic NSAID Drug Induced Oxidative Stress. *Chem. Biol. Interact*. **2002**, 142 (1–2), 25–41. https://doi.org/10.1016/S0009-2797(02)00052-2.
36. Boelsterli, U. Diclofenac-Induced Liver Injury: A Paradigm of Idiosyncratic Drug Toxicity. *Toxicol. Appl. Pharmacol.* **2003**, 192 (3), 307–322. https://doi.org/10.1016/S0041-008X(03)00368-5.
37. Marsh, D. Spin-Label Electron Paramagnetic Resonance Spectroscopy; CRC Press: Boca Raton, FL, USA, 2020.
38. Erilov, D.A.; Bartucci, R.; Guzzi, R.; Shubin, A.A.; Maryasov, A.G.; Marsh, D.; Dzuba, S.A.; Sportelli, L. Water concentration profiles in membranes measured by ESEEM of spin-labeled lipids. J. Phys. Chem. B **2005**, 109, 12003–12013.
39. Milov, A.D.; Samoilova, R.I.; Shubin, A.A.; Grishin, Y.A.; Dzuba, S.A. ESEEM measurements of local water concentration in D_2O-containing spin-labeled systems. Appl. Magn. Reson. **2008**, 35, 73–94.
40. Dzuba, S.A.; Marsh, D. ESEEM of Spin Labels to Study Intermolecular Interactions, Molecular Assembly and Conformation. In A Specialist Periodic Report, Electron Paramagnetic Resonance; Gilbert, C., Chechik, V., Murphy, D.M., Eds.; RSC Publishing: Cambridge, UK, 2015; Volume 24, pp. 102–121.
41. Konov, K.B.; Isaev N.P.; Dzuba, S.A. Glycerol penetration profile in phospholipid bilayers measured by ESEEM of spin-labelled lipids, Mol. Phys. **2013**, 111, 2882-28863. https://doi.org/10.1080/00268976.2013.796416
42. Shapiro, A. B.; Skripnichenko, L. N.; Pavlikov, V. V; Rozantsev, E. G. Synthesis of Nitroxyl Radicals Based on 4-Ethynyl-4-Hydroxy-2,2,6,6-Tetramethylpiperidine. *Russ. Chem. Bull.* **1979**, 28, 140–148. https://doi.org/10.1007/BF00925412.
43. Tanwar, L.; Börgel, J.; Lehmann, J.; Ritter, T. Selective C-H Iodination of (Hetero)Arenes. *Org. Lett.* **2021**, 23 (13), 5024–5027. https://doi.org/10.1021/acs.orglett.1c01530.
44. Dalai, P. G.; Palit, K.; Panda, N. Generation of Dimethyl Sulfoxide Coordinated Thermally Stable Halogen Cation Pools for C–H Halogenation. *Adv. Synth. Catal.* **2022**, 364 (5), 1031–1038. https://doi.org/10.1002/adsc.202101291.
45. Smorygina A.S.; Golysheva E. A.; Dzuba S.A. Clustering of Stearic Acids in Model Phospholipid Membranes Revealed by Double Electron−Electron Resonance. *Langmuir* **2021**, 37, 13909–13916. https://doi.org/10.1021/acs.langmuir.1c02460

46. Unguryan, V.V.; Golysheva, E.A.; Dzuba, S.A. Double electron-electron resonance of spin-labeled cholestane in model membranes: evidence for substructures inside the lipid rafts, J. Phys. Chem. B, **2021**, 125, 33, 9557–9563, DOI: 10.1021/acs.jpcb.1c05215
47. Kashnik, A.S.; Baranov, D.S.; Dzuba, S.A. Ibuprofen in a lipid bilayer: nanoscale spatial arrangement. *Membranes* **2022**, 12, 1077. https://doi.org/10.3390/membranes12111077
48. Kashnik, A.S.; Syryamina, V.N.; Biondi, B.; Peggion, C.; Formaggio, F.; Dzuba, S.A. DEER/PELDOR Study of the Effect of Extremely Low Concentrations of the Antimicrobial Peptide Chalciporin A on the Membrane Lipid Organization. Appl. Magn. Reson. 2023, **54**, 401–414. https://doi.org/10.1007/s00723-023-01526-x