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Synthetic Strategies for the Modification of Diclofenac

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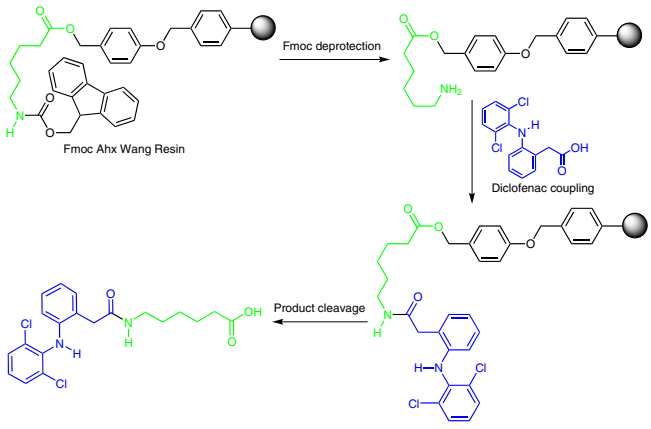

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Abstract For many heterogeneous sensor applications as well as the synthesis of hapten antigens to produce antibodies, protein conjugates of the target substance are essential. A requirement is that the target substance already offers or is modified to contain a functionality that allows for coupling to a protein, that is, an amino acid residue. Ideally, to avoid shielding of the compound by the carrier protein, a sufficient distance to the protein surface should be provided. With its carboxyl function diclofenac (DCF) allows for direct binding to lysine residues after in situ synthesis of the NHS ester. One problem is that diclofenac as free acid tends to autocondensation, which results in low yields. Here we describe the 'insertion' of a C6 spacer via synthesis of the amide with 6-aminohexanoic acid. To carry out the reaction in solution, first the methyl ester of the amino acid had to be produced. Due to otherwise low yields and large cleaning efforts, solid-phase synthesis on Fmoc Ahx Wang resin is recommended. The crude product is mainly contaminated by cleavage products from the resin which were removed by chromatography. The structure of the highly pure hapten was completely determined by nuclear magnetic resonance (NMR) spectroscopy.

Key words diclofenac, hapten synthesis, solid-phase synthesis, spacer introduction, amide linkage

Diclofenac (DCF, Figure 1) is a pharmacologically active compound which is used as a nonsteroidal anti-inflammatory drug against fever, inflammation, rheumatic diseases, and pain. Its mode of action is based on inhibition of cyclooxygenase-2. Of all drugs, DCF is among the most commonly used and accordingly also detected regularly in the aquatic environment. In Germany alone, every year up to 80 t are sold.[1] Around 65–70% of ingested DCF is excreted in urine.[2] Topical use leads to wash-off of the compound while showering. Due to incomplete removal in wastewater treatment plants (WWTP, depending on the study between 17–75%[3]), residues in surface water and drinking water have been detected. In India, the accumulation of DCF has led to a mass extinction of vultures.[4]

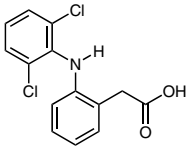

Figure 1 Diclofenac (DCF)

Due to these circumstances DCF was introduced to the first Watch List of the EU Water Framework Directive.[5] The proposed limit of 100 ng/L specified for WWTP effluents is clearly exceeded according to several studies.[2,6] Current detection in environmental samples is carried out mainly by GC–MS and LC–MS methods. Another approach is the detection by immunoassays. These are cost-effective methods, require less time, and enable for the high-throughput analysis of many samples. Measurement at the point of sampling is possible, too, as immunosensors[7] and dipstick assays[8] are becoming widely spread. There are antibodies

against a variety of pharmacologically active substances, for example, caffeine[9], carbamazepine,[10] isolithocholic acid,[11] sulfonamides,[12] 2,4,6-trinitrotoluene (TNT),[13] picoxystrobin,[14] cocaine,[15] and bisphenol A.[16] A polyclonal antidiclofenac antibody is available, with which already measurements were made in environmental samples.[17] Recently, the development of a monoclonal antibody was published.[18] It is the first application of a monoclonal antidiclofenac antibody in environmental analysis. However, already in 2010, different monoclonal antibodies against diclofenac and a number of its metabolites were developed for medical research. Yet, only the production of IgE-containing polyclonal antisera was published.[19] All these antibodies are based on immunogens generated by the direct coupling of diclofenac to the respective carrier protein via its carboxyl function. However, through the introduction of a spacer, more selective and possibly antibodies with higher affinity could be expected. An optimum of the length of alkyl spacers is often achieved with six carbon atoms.[20] As a spacer of choice 6-aminohexanoic acid is selected. Via an amide linkage between the spacer and DCF the hapten **H1** DCF-Ahx (Figure 2) is created. Because of the carboxylic acid group **H1** can be bound to proteins under the same conditions as DCF. An alternative is hapten **H2**, an ester of DCF and 2-aminoethanol (Figure 3). **H2** allows the coupling via an amino function.

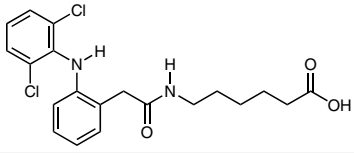

Figure 2 Structure of hapten DCF-Ahx (**H1**)

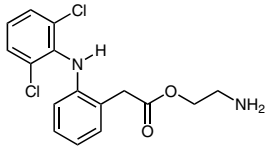

Figure 3 Structure of hapten **H2**

Commercially, DCF is available as the sodium salt. If DCF is converted into the free carboxylic acid or an activated form, cyclisation is the result. The cyclisation product (DCF lactam, Figure 4) was found in environmental samples, too.

Because of the cyclization side reaction chemical modifications, such as the syntheses of novel diclofenac haptens, are very difficult. This paper is intended to provide an overview of successful syntheses[21] resulting from some general problem solving approaches and the application of the products.

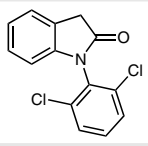

Figure 4 Cyclisation product of DCF: DCF lactam

The goal of the experiments was the synthesis of an amide of diclofenac and 6-aminohexanoic acid **H1** as well as an ester of diclofenac and 2-aminoethanol **H2**. The haptens can be synthesized under homogeneous conditions. Alternatively, it is possible to produce **H1** in a solid-phase synthesis. A modification of diclofenac proved to be extremely difficult because of the very rapidly occurring cyclization under acidic conditions or after activation with several activation regents, like NHS, hydroxybenzotriazole (HOBT), or oxalyl chloride.

Synthesis of **H1** was performed in DMF. Ahx is not soluble in DMF. This problem was circumvented by using an Ahx methyl ester. Initial experiments aimed at activation of DCF with NHS, 1-hydroxybenzotriazol (HOBT), or the synthesis of various mixed anhydrides, followed by reaction with AhxMe. Although a product formation was detected by HPLC–MS, the reaction proceeded in low yield. Moreover, after two-stage purification by column chromatography on silica gel and reversed-phase chromatography contaminants were still found in an LC–UV/Vis run (Figure 5). The difference of 0.2 min in the UV trace and the MS trace is caused by the transfer of sample from the HPLC to the MS detector.

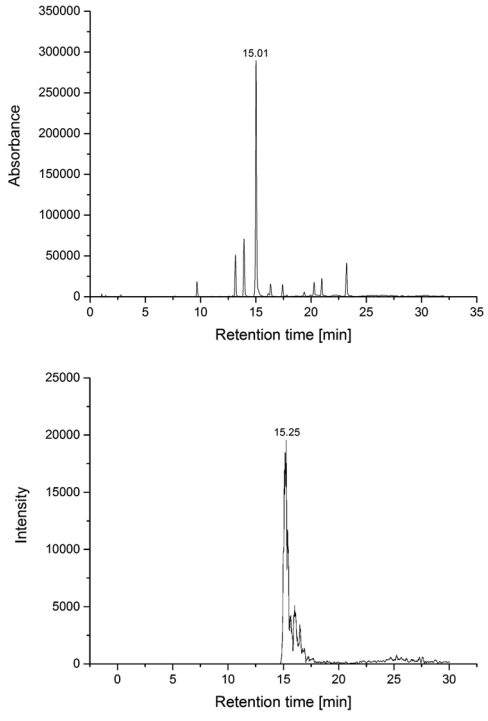

Figure 5 HPLC chromatogram of DCF-AhxMe, UV/Vis trace (upper), MS trace at m/z = 423 (lower)

Because DCF already forms a methyl ester, when it is acidified in methanolic solution, it has been tried to make this property employable for hapten synthesis of **H2**. For this purpose, DCF was dissolved in 2-aminoethanol. The solution was then acidified. HPLC–MS analysis confirmed product formation, but in low yield (Figure 6). Higher amounts in starting materials could provide higher yields. However, the purification proved to be difficult, which is why this synthesis must be assessed overall as ineffective.

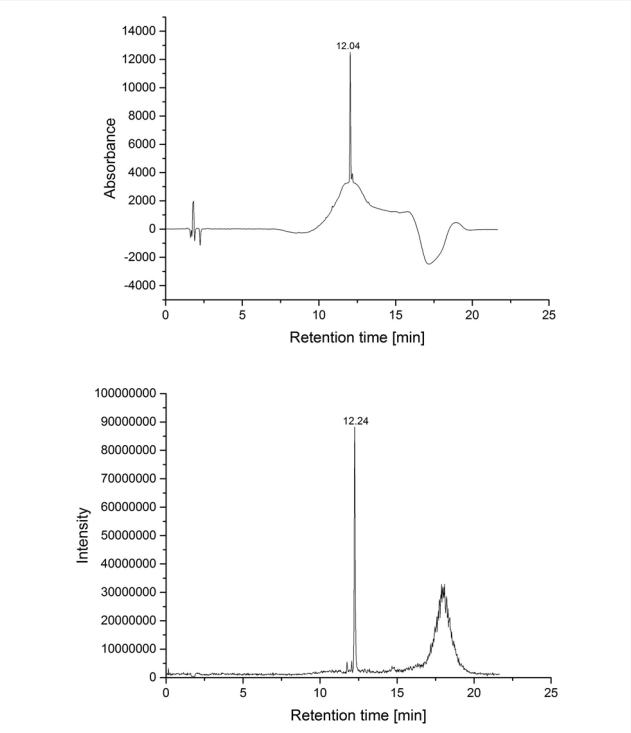

Figure 6 HPLC chromatogram of **H2**, DAD trace (upper) and MS trace at *m/z* = 339 (lower)

Another approach is the reaction with Grignard- or lithium-activated spacers, where diclofenac does not require activation. Diclofenac must be available as methyl ester as starting material. The DCF methyl ester is easy to synthesize. However, the reaction with an organometallic compound leads again only to the cyclic product DCF lactam. It can be assumed that the ester is cleaved intramolecularly under such basic conditions.

As described, a methyl ester of **H1** could be synthesized in homogeneous solution. Because of the impurities and a required ester hydrolysis, this synthesis route was ineffective. For this reason, solid-phase synthesis was chosen, as here impurities can be washed from the resin. Due to the low loading, 5 g of resin were used for each batch to be able to provide sufficient product. Various resins have been tested in different reaction chambers. First, the reaction was carried out in glass bottles, followed by filtration of the resin. However, these steps were too cumbersome, which is why a glass column was used as a reactor. Reactants and wash solutions can be easily rinsed from the resin. The duration of the synthesis depends strongly on the type and porosity of the resin, since the frit of the column may clog, which results in very long rinsing times. As optimum resin Fmoc Ahx Wang Resin was determined. Advantages are on the one hand, that an appropriate C6 spacer is already bound to the resin, so only the coupling of diclofenac is required and the consumption of solvents is drastically reduced. Moreover, all solutions can be rapidly withdrawn from the resin. All resins in common is that together with the product impurities are cleaved from the resin, which cannot be completely removed under vacuum and act as solvent for the product. For this reason, a preliminary purification on silica gel is required. The final purification was carried out by HPLC. The yield was 0.4%, reproducibly. Using 5 g resin, 2.3 mg hapten DCF-Ahx could be obtained. This amount is sufficient to synthesize protein conjugates for immunization and development of an ELISA. In addition, NMR spectra can be produced for structure confirmation. As can be seen in the chromatogram with diode array detection (DAD), the product is slightly contaminated (Figure 7).

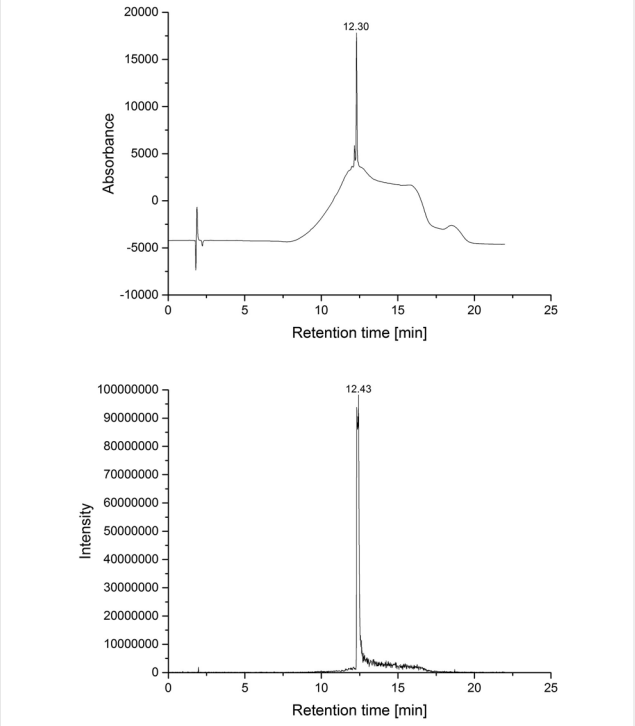

Figure 7 HPLC chromatogram of DCF-Ahx, DAD (upper) and MS trace at *m/z* = 409 (lower)

This contamination could not be removed and leads to additional peaks in the NMR spectrum. Nevertheless, the structure could be unambiguously confirmed by NMR spectroscopy. Furthermore, it had no effect on protein coupling.

The main reason for the low yield is the aforementioned cyclization of DCF after activation. Also, synthesis in the microwave reactor did not improve the yield. A new approach for synthesis of DCF-Ahx was the activation with NHS. This activation is often used in peptide modifications. In classical solid-phase synthesis the activation and coupling is done in situ in one hour. Activation with NHS for DCF-Ahx needs to be conducted overnight. The long activation time reduces side reactions, like cyclization, and leads to higher yields. However, the coupling must be done under argon atmosphere. This approach gave 138 mg hapten DCF-Ahx. In addition to the higher yield the product was cleaner. Only a purification step by column chromatography on silica was necessary. The HPLC chromatogram (Figure 8) and NMR spectra (Figure 9 and Figure 10) show 100% purity. The high amount of product allows to determine the structure. Parallel to our work another group synthesized an amide of DCF and glycine (DCF-Gly) in similar ways by solid-phase synthesis.[22]

As described before, the reactivity of diclofenac allows only very limited scope for syntheses. The added value to the known synthesis of DCF-Gly[22] is that our synthesis of DCF-Ahx (aminohexanoic acid amide of diclofenac) provides via solid-phase synthesis a pure product in high yield by avoiding cyclization. The product enables, due to the C6 spacer, for protein conjugates with better exposure of the diclofenac moiety, an important feature with antigens for antibody production.

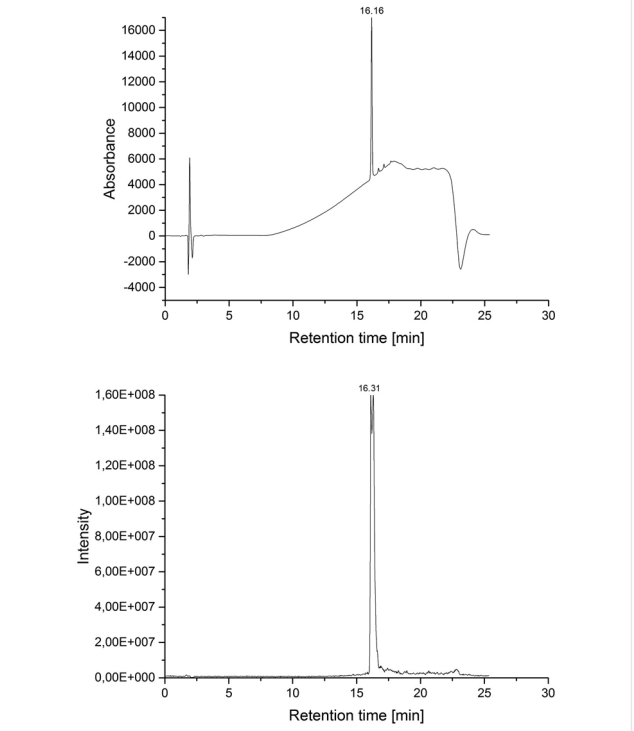

Figure 8 HPLC chromatogram of DCF-Ahx (**H1**), DAD (upper) and MS trace at m/z = 409 (lower)

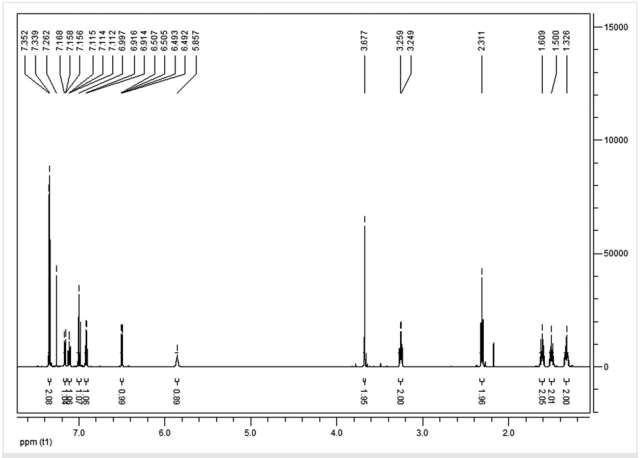

Figure 9 ^{1}H NMR spectrum of DCF-Ahx (**H1**)

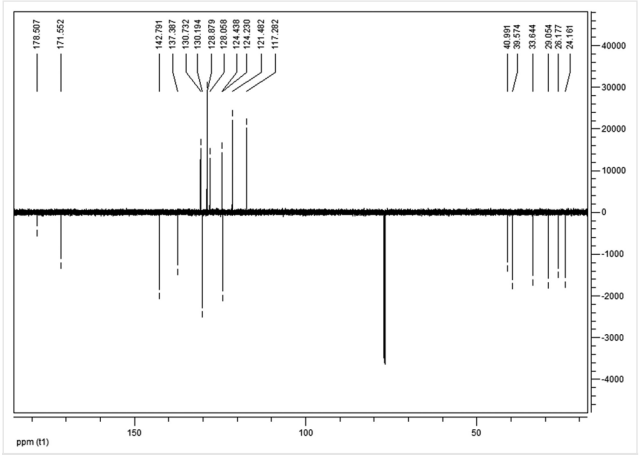

Figure 10 ^{13}C NMR spectrum of DCF-Ahx (**H1**)

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(21) **Reagents**

All standard chemicals and reagents were purchased from Sigma-Aldrich (Steinheim, Germany). All solvents were chromatography grade. Diclofenac sodium salt, 1-*N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), piperidine, trifluoroacetic acid (TFA), 1-hydroxybenzotriazol (HOBT), triisopropylsilane (TIS), chlorotrimethylsilane, *N*,*N*-dimethylformamide (DMF), dichloromethane (CH_2Cl_2), 6-aminohexanoic acid (Ahx), cyclohexane, and ethyl acetate (EtOAc) were also purchased from Sigma-Aldrich. Fmoc Ahx Wang resin was supplied by Iris Biotech (Marktredwitz, Germany). 2-Aminoethanol and sulfuric acid (98%) were purchased from J.T. Baker (Griesheim, Germany). Piperidine was supplied by Roth (Karlsruhe, Germany). For HPLC the following solvent compounds have been used: ammonium acetate (NH_4Ac, analytical grade, 99.3%, Fischer Chemicals, Zurich, Switzerland), acetic acid glacial (AcOH, analytical reagent grade, Fischer Chemicals) and methanol (MeOH, HPLC gradient grade, J.T. Baker). For production of ultrapure water a water purification system (Milli-Q Synthesis A10, Merck Millipore, Schwalbach, Germany) was employed.

Instruments

HPLC-MS studies were performed on an Agilent 1260 Infinity LC system with binary pump, degasser, autosampler, column heater, and UV detector. The chromatographic separation was carried out on a Kinetex X_B-C18, 2.6 µm, 150 mm × 3 mm (Phenomenex, Aschaffenburg, Germany) analytical LC column with UHPLC C18, 3 mm (Phenomenex) column guard. As mobile phases Milli-Q water with 10 mM NH_4Ac and 0.1% (v/v) acetic acid (A) and MeOH with 10 mM NH_4Ac and 0.1% (v:v) AcOH (B) were used. The system was run with a flow rate of 350 µL/min and a column heater temperature of 50 °C. An elution gradient was applied, starting with 80% A for the first 3 min. Within 5 min A is reduced to 5% and maintained at this level for a further 4 min. Then A is again increased to 80% within 2 min and held for 8 min to re-equilibrate the column. 10 µL sample was injected. The mass determination was performed on an ABSciex 6500 triple quad mass spectrometer. For electrospray ionization (ESI) the positive ionization mode was selected. For purification of crude products an Agilent 1200 Series LC System was used with the same method and an identical column. 30 µL sample was injected, and fractions were collected from 12.25–12.45 min. Products were dried in a stream of nitrogen. Structure determination was made by registering ^{1}H NMR and ^{13}C NMR spectra under standard conditions with a Bruker Avance 600 MHz NMR spectrometer operating at 600.2 MHz. The substances had been dissolved in $CDCl_4$. Trimethylsilane was used as reference.

Synthesis of 6-{[2-(2,6-Dichlorophenylamino)phenyl]acetamido}-hexanoic Acid (DCF-Ahx)

5 g of Fmoc Ahx Wang resin (degree of loading 0.3 mmol/g) were transferred to a glass column and swollen in DMF for 1 h. For optimum mixing the glass column was shaken. For Fmoc deprotection DMF/piperidine (4:1, v/v) was added and shaken for 10 min. After this time the resin was washed twice with DMF and twice with CH_2Cl_2. The resin was dried and transferred in an argon atmosphere. The resin was loaded with 2 g DCF (sodium salt), 806 mg of NHS, and 1.4 g DCC in 50 mL dry DMF. The reaction mixture was shaken overnight. The next day, the resin was washed four times with MeOH, twice with DMF and twice with CH_2Cl_2. Cleavage of the product was done by adding TFA/TIS/H_2O (95:2.5:2.5, v/v/v). After 2 h reaction time, the resin was extracted once with TFA. The dry resin was washed with TFA again. The combined cleavage and extraction solutions were concentrated at 75 °C on a rotary evaporator. The resulting yellow oil was purified by column chromatography on silica gel using the mobile phase cyclohexane/EtOAc (1:1, v/v). Every fifth fraction was analyzed by HPLC–MS. The fractions which contained the desired purified product were combined and the solvent evaporated on a rotary evaporator. 138 mg (22.5%) of a white solid were obtained.

Analytical Data

^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.33 (m, 2 H, CH_2), 1.52 (m, *J*1 = 7.58 Hz, *J*2 = 7.58 Hz, *J*3 = 14.96 Hz, 2 H, CH_2), 1.62 (m, *J*1 = 7.43 Hz, *J*2 = 7.43 Hz, *J*3 = 15.13 Hz, 2 H, CH_2), 2.31 (t, *J*1 = 7.36 Hz, *J*2 = 7.36 Hz, 2 H, CH_2), 3.25 (q, *J*1 = 7.11 Hz, *J*2 = 13.16 Hz, 2 H, CH_2), 3.67 (s, 2 H, CH_2), 5.85 (m, *J*1 = 5.22 Hz, *J*2 = 5.22 Hz, 1 H, ArH), 6.50 (dd, *J*1 = 1.01 Hz, *J*2 = 8.06 Hz, 1 H, ArH), 6.91 (t, *J*1 = 1.19 Hz, *J*2 = 7.42 Hz, *J*3 = 7.42 Hz, 1 H, ArH), 6.99 (m, *J*1 = 8.07 Hz, *J*2 = 8.07 Hz, 1 H, ArH), 7.11 (m, *J*1 = 1.38 Hz, *J*2 = 7.50 Hz, 1 H, ArH), 7.15 (m, *J*1 = 1.38 Hz, *J*2 = 7.50 Hz, 1 H, ArH), 7.33 (d, *J* = 8.06 Hz, 2 H, ArH) ppm.

^{13}C NMR (125 MHz, $CDCl_3$): δ = 24.16, 26.17, 29.05, 33.64, 39.57, 40.99 (6 × CH_2), 117.28, 121.48, 124.23 (3 × CH_{Ar}), 124.43 (qC), 128.05, 128.87 (2 × CH_{Ar}), 130.19 (qC), 130.73 (CH_{Ar}), 137.38, 142.79 (2 × qC), 171.55 (CO), 178.50 (COOH) ppm.

Synthesis Trial for 2-Aminoethyl-2-[2-(2,6-dichlorophenylamino)phenyl]acetate

100 mg DCF (sodium salt) was dissolved in 3 mL 2-aminoethanol. Carefully 1 mL sulfuric acid was added at 0 °C. The reaction mixture was stirred for 2 h. After this time the reaction mixture was analyzed by HPLC–MS.

Synthesis of DCF Methyl Ester (DCFMe)

1 g DCF (sodium salt) was dissolved in 7 mL DMF. After addition of 7 mL MeI, the reaction mixture was stirred overnight. On the next day, the solvent was removed under vacuum. The solid was suspended in EtOAc. The organic solution was washed with water three times. The organic phase was dried with $MgSO_4$. After filtration, EtOAc was removed under vacuum. 893 mg (90%) of a yellow solid was obtained.

Synthesis of Ahx Methyl Ester (AhxMe)

1 g Ahx was dissolved in 2 mL chlorotrimethylsilane. After addition of 8 mL MeOH, the reaction mixture was stirred overnight. On the next day, the solvent was removed under vacuum.

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