

Synthesis of a lysosome-targeting aminoferrocene-based prodrug **NCure2**

Maximilian Dill, Attia Dina, Mokhir Andriy*

Friedrich-Alexander-University Erlangen-Nürnberg (FAU) Department of Chemistry and Pharmacy Organic Chemistry Chair II, Nikolaus-Fiebiger-Str. 10, 91058 Erlangen (Germany)

Email: Andriy.Mokhir@fau.de

Received mm-dd-yyyy

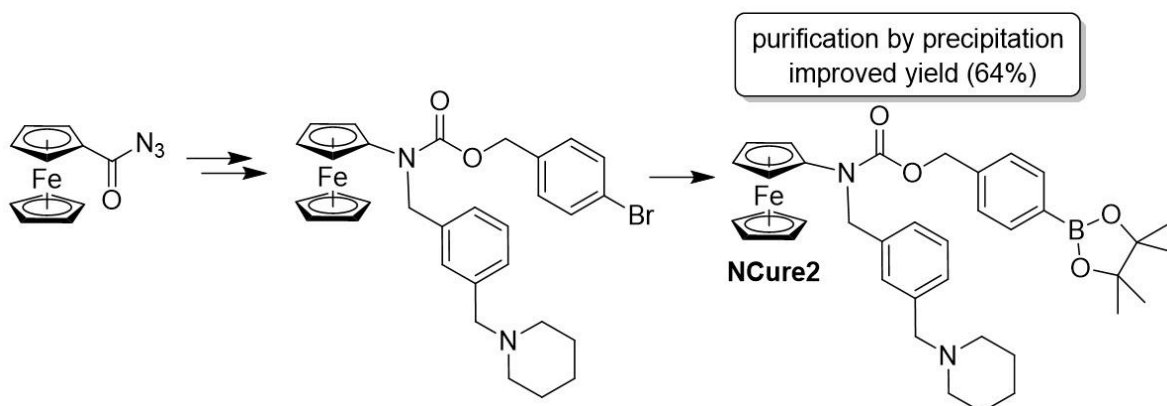
Accepted mm-dd-yyyy

Published on line mm-dd-yyyy

Dates to be inserted by editorial office

Abstract

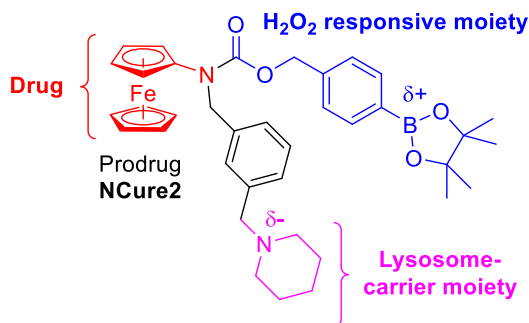
Prodrugs can achieve targeting of cancer cells. Therefore, their application can improve the therapy of cancer diseases. The best aminoferrocene-based prodrug developed in the group of Mokhir is 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl (3-(piperidin-1-ylmethyl)benzyl) (ferrocenyl)carbamate, **NCure2**. It targets lysosomes of cancer cells, where it is activated by H₂O₂. Due to the presence of basic (amine) and Lewis acidic (boronic acid pinacol ester) moieties in **NCure2**, the previous synthesis is low yielding and purification is laborious. In this work we report on a significantly improved synthetic protocol for preparation of HCl salt of **NCure2** by introducing the boronic acid moiety in the last synthetic step *via* Miyaura borylation conditions that allows the facile purification of the final product by its precipitation. This new approach makes **NCure2**·HCl more synthetically accessible that will facilitate its further pre-clinical and clinical studies.



Keywords: Anticancer prodrug, Aminoferrocene, Synthesis, Scale up, C-B Couplin

Introduction

One possible approach for precise targeting of cancer cells is based on the prodrug concept. A prodrug is an inactive compound, which can be activated in cancer specific environment with formation of an active drug.¹ Since healthy cells do not possess such an environment, the prodrug remains inactive in these cells. For example, the group of Mokhir, as well as others, have explored differences in the level of H₂O₂ in cancer and healthy cells.²⁻⁶ H₂O₂ is the most abundant intracellular reactive oxygen species (ROS). One of the most successful H₂O₂-responsive prodrugs activated by H₂O₂, which has been developed in the group of Mokhir up to date, is 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl (3-(piperidin-1-ylmethyl)benzyl) (ferrocenyl)carbamate,



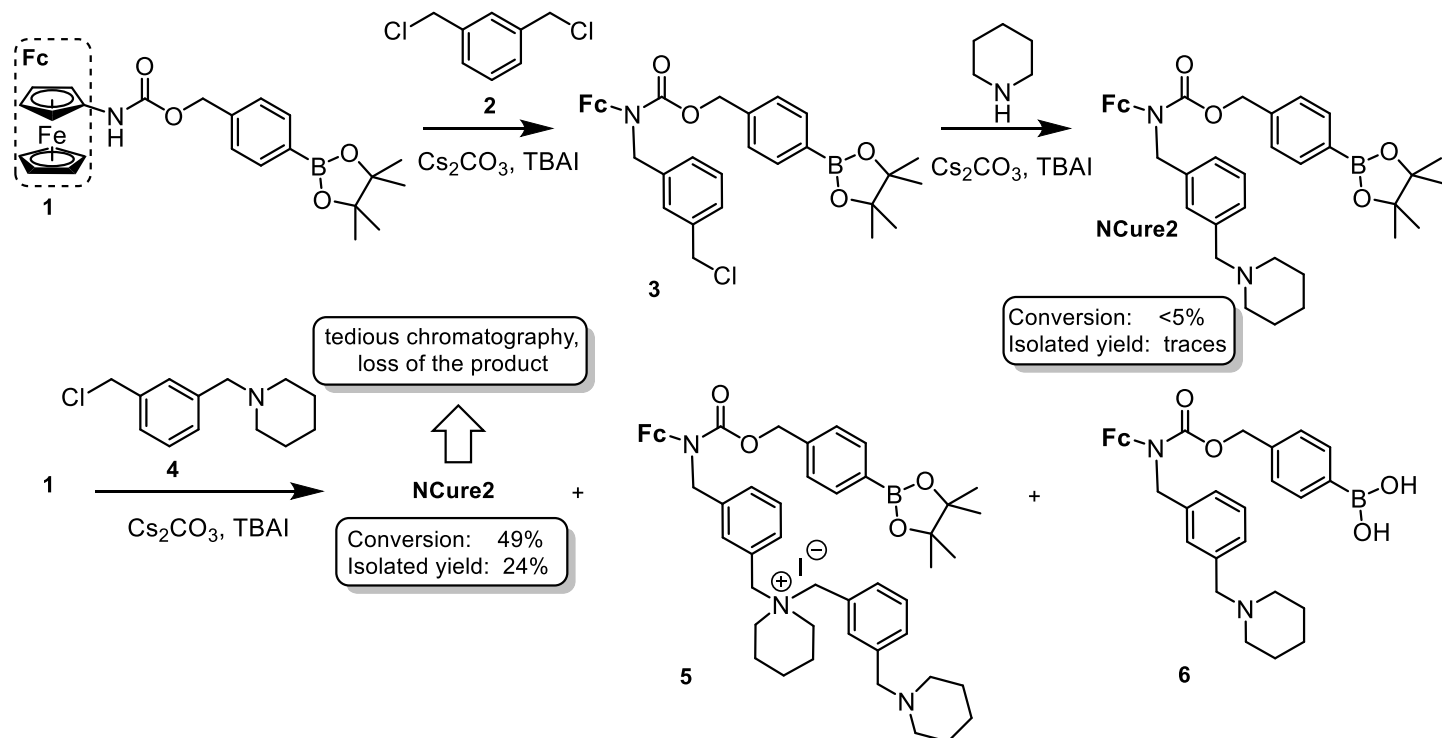
called **NCure2**.⁷ Its chemical structure is shown in Figure 1.

Figure 1. A structure of prodrug **NCure2** highlighting with different colors the moieties important for its activity.

NCure2 contains three elements important for its activity. First, a basic piperidine moiety (magenta colored), which is a lysosomal carrier. Second, a Lewis acidic arylboronic acid pinacol ester (blue colored), which is cleaved in the presence of cancer specific levels of H₂O₂ and third, an aminoferrocene drug (red colored), which is released upon the cleavage of the arylboronic acid moiety and induces the oxidative stress in cancer cells leading to their death. **NCure2** exhibits excellent antitumor activity *in vitro* in a variety of cancer cell lines derived from blood, ovary, lung and prostate and in some primary cells, e.g., chronic lymphocytic leukemia (CLL) cells. Importantly, it is also active *in vivo* in the Nemeth-Kellner-lymphoma (NK/Ly) model of murine cancer. **NCure2** is not toxic towards healthy cell line SBLF9 and primary cells including neutrophils, monocytes, B and T cells at the therapeutically active concentrations.

Currently known methods of synthesis of this interesting prodrug are outlined in Scheme 1. The first method is based on *N*-alkylation of 4-(ferrocenylaminocarbonyloxymethyl)phenylboronic acid pinacol ester (**1**) with 1,3-bis(chloromethyl)benzene (**2**) in the presence of Cs₂CO₃, followed by amination of the resulting product **3** by using piperidine. These reactions can be conducted either one after another with isolation of the intermediate **3** or sequentially in one pot. In both cases the conversion of **1** to **NCure2** is less than 5%. The isolation is tedious and leads to only traces of the desired product due to the presence of the large number of unidentified side products in the reaction mixture. We improved the initial protocol by coupling of pre-synthesized *N*-(3-(chloromethyl)benzyl)piperidine **4** to the starting material **1**. The conversion of **1** to **NCure2** is 49% under these optimized conditions. Unfortunately, overalkylated (**5**), hydrolyzed **NCure2** (**6**) as well as other unidentified products are also formed. Some of these side products are difficult to separate from **NCure2** which reduces the

yield of the isolated product to 24%. The latter optimized protocol was used extensively by our group for the preparation of 10 – 100 mg of **NCure2** in one run that was sufficient for the majority of *in vitro* and preliminary *in vivo* studies. However, for further pre-clinical evaluation of this prodrug, e.g. *in vivo* studies in different cancer models in mice and rats, larger quantities (>100 mg) are required. The current work addresses this need by reporting an improved protocol that should enable facile larger scale synthesis of **NCure2**. The key advancement is the introduction of the boronic acid pinacol ester moiety during the last synthetic step, which occurs with almost quantitative conversion thereby eliminating the need for the extensive purification of the prodrug.

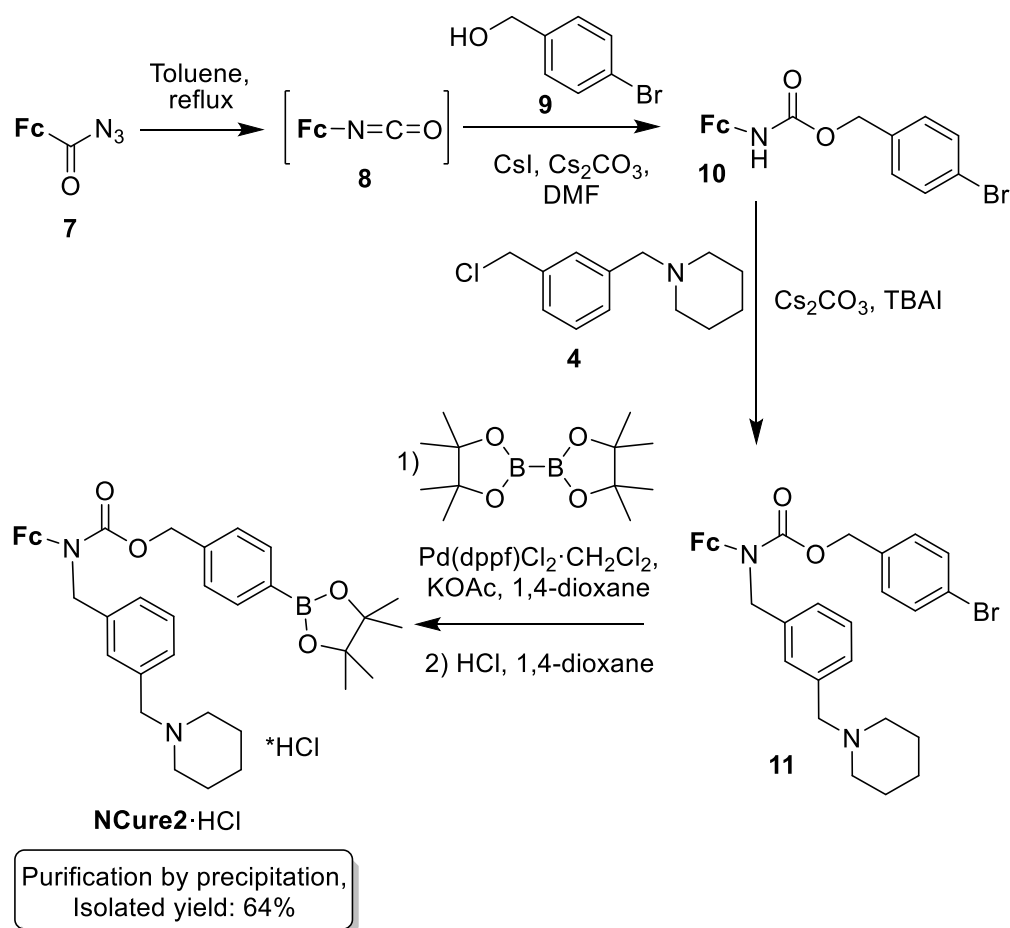


Scheme 1. Previous synthetic routes to **NCure2**.⁷

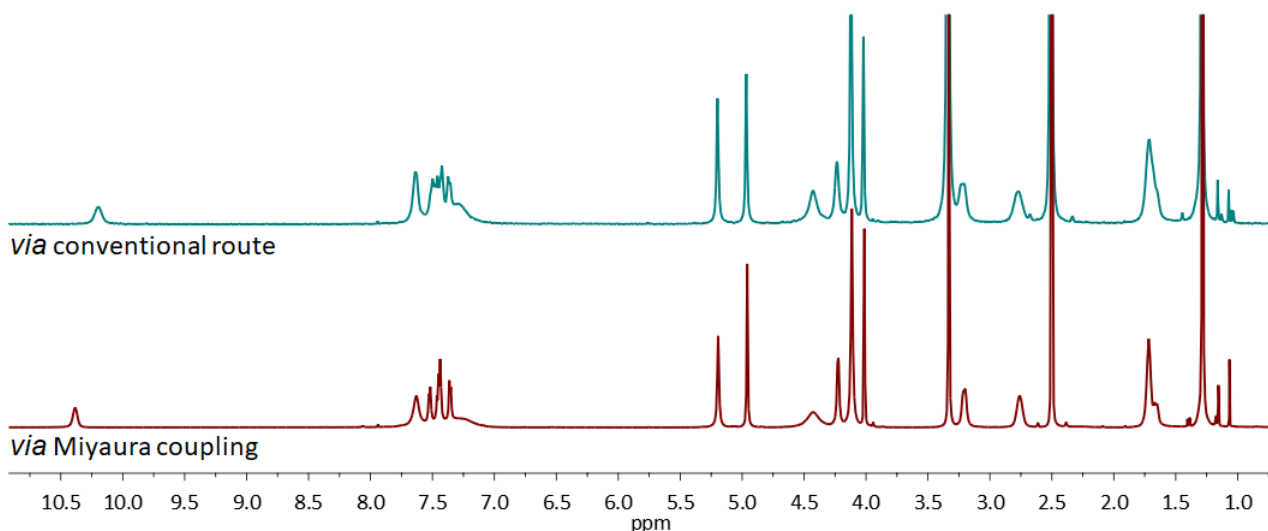
Results and Discussion

NCure2 is a polar compound due to the presence of a basic moiety (*N*-alkylated piperidine) and a Lewis acidic moiety (arylboronic acid pinacol ester). It is challenging to purify this compound even from simple mixtures by using flash chromatography, because it diffuses on the column to a broad band under all neutral, basic or acidic conditions, presumably owing to on column hydrolysis followed by strong absorption of the boronic acid product of the solid phase. Furthermore, purification of **NCure2** on silica often leads to the on-column hydrolysis followed by strong, partially irreversible absorption of the boronic acid product **6** (Scheme 1) that contributes to the product loss. The latter property has been also described for other arylboronic acid esters.⁸⁻¹⁰ To address these challenges, we planned the synthesis of **NCure2** in a way that the last step is high yielding and produces side products which can be removed by non-chromatographic methods. The synthetic approach is outlined in Scheme 2. In contrast to the previous methods (Scheme 1), where the boronic acid pinacol ester was present from the very beginning (starting material **1**), the latter fragment is introduced in the last step in the new synthesis (Scheme 2). We selected for this critical step the Miyaura borylation, since its mild conditions are compatible with other functional groups present in **NCure2**. In particular, we started with Curtius rearrangement including the conversion of ferrocenecarboxylic azide (**7**) to ferrocenylisocyanate **8** in refluxing

toluene, followed by trapping of the latter compound with 4-bromobenzyl alcohol (**9**) in the presence of Cs_2CO_3 with formation of 4-bromobenzyl (ferrocenyl)carbamate (**10**) with the excellent yield of 87%. Curtius reactions with azide **7** are well known and have been previously applied to a vast variety of alcohols.¹¹⁻¹³ Next, intermediate **10** was reacted with known *N*-(3-(chloromethyl)benzyl)piperidine (**4**)⁷ to obtain precursor **11**. This compound was purified by silica column chromatography using the trimethylamine-containing eluent mixture to keep the aliphatic amine moiety in the deprotonated state to minimize strong absorption and diffusion of the band. The purification under these conditions was efficient and yielded 65% of **11**. In the final step, the boronate ester was introduced *via* Miyaura borylation using $\text{Pd}(\text{dppf})\text{Cl}_2 \cdot \text{CH}_2\text{Cl}_2$ as catalyst in 1,4-dioxane together with $(\text{BPin})_2$ and KOAc. The mixture was degassed with N_2 , sealed in a microwave tube and heated to 80 °C for 19 h. The product mixture was filtered through Celite® with charcoal topping, which removes palladium residues. Afterwards, the product **NCure2** was transformed to its HCl salt by addition of a HCl solution in 1,4-dioxane. The solution was filtered once more to remove remaining Pd nanoparticles. Then, isopropanol was added, leading to precipitation of **NCure2**·HCl, which was filtered, washed and dried. Through precipitation, 64% yield of **NCure2**·HCl were obtained. Byproducts of the reaction remained in the mother liquor. The obtained product was unambiguously identified by high resolution, atmospheric pressure photoionization mass spectrometry (HRMS APPI) as well as ^1H and ^{13}C NMR spectroscopy (Figures S5 & S6, supporting information (S/)) and elemental analysis. The purity of the product was confirmed by thin layer chromatography in three different eluents.



Scheme 2. An improved method of synthesis of the prodrug **NCure2**·HCl.

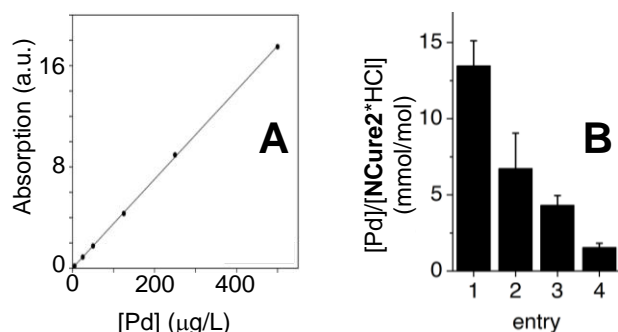


The new method gives rise to **NCure2**·HCl and the conventional method gives rise to **NCure2** (Scheme 1). To compare qualities of the products obtained *via* these approaches, we converted the **NCure2** obtained with the conventional route to its salt by adding HCl in 1,4-dioxane, followed by the precipitation of **NCure2**·HCl with isopropanol. According to ^1H NMR spectroscopy, both samples were found to be practically identical (Figure 2).

Figure 2. ^1H NMR spectra of **NCure2**·HCl synthesized by the conventional route⁷ (upper trace) and Miyaura borylation (lower trace).

We obtained 453 mg of analytically pure **NCure2**·HCl in a single run without the need for tedious chromatographic purification in the last step. This protocol was repeated three times giving rise to similar yields of **NCure2**·HCl.

Since palladium, palladium nanoparticles and ions can exhibit toxicity towards cells,¹⁴⁻¹⁶ we tested the content of Pd in the samples of **NCure2**·HCl obtained from three independent syntheses, conducted by two different



researchers. We used atomic emission spectroscopy (AES), since it is a highly sensitive method. Under our experimental settings, AES allowed the accurate detection of as little as 25 $\mu\text{g/L}$ Pd in solution (235 nm) as it is apparent from the calibration of the AES signal intensity *versus* the concentration of Pd(II) (Figure 3A).

Figure 3. Atomic emission spectroscopy (AES) data. **A:** A calibration plot of the AES response (OY axis: absorbance, expressed in arbitrary units, a.u.) *versus* the concentration of $\text{Pd}(\text{NO}_3)_2$ solution in HNO_3 (OX axis: [Pd]). **B:** Content of Pd in samples of **NCure2**·HCl prepared by the new method described in this paper (entries

1-3). Entry 4: a negative control. Conventionally synthesized **NCure2**·HCl (Scheme 1) was used as a negative control, since no palladium-containing reagents were used during its synthesis.

The data on the Pd content in the **NCure2**·HCl samples are provided in Figure 3B. We observed that the palladium concentration is increased statistically significantly in the samples of **NCure2**·HCl prepared by the new method when compared to the sample of the prodrug prepared by the conventional method (a negative control, entry 4): Student's t test, entry 1: $p < 0.001$; entry 3: $p < 0.05$; entry 3: $p < 0.01$. The absolute palladium amount is 0.04 – 0.19% Pd in the samples of the prodrug.

To exclude that the Pd traces will have some effect on the biological activity of the **NCure2**·HCl, we investigated its cytotoxicity towards human ovarian cancer (A2780) cells. We used (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide probe (MTT) to determine the number of viable A2780 cells. Conventionally synthesized **NCure2**·HCl (Scheme 1) was used as a control lacking Pd traces (Figure 4). We found that there is no difference in the anticancer effect of **NCure2**·HCl prodrugs prepared by the different methods: inhibitory concentrations (IC_{50} 's) for the **NCure2**·HCl obtained by the new method (from $5.10 \pm 0.02 \mu\text{M}$ to $5.62 \pm 0.50 \mu\text{M}$, entries 1-3) are the same as the IC_{50} for the **NCure2**·HCl obtained by the conventional method ($4.97 \pm 1.13 \mu\text{M}$, entry 4; Student's t test, $p > 0.05$ for all pairs). These data confirm that the Pd traces present in the samples obtained by the new method do not modulate their anticancer activity.

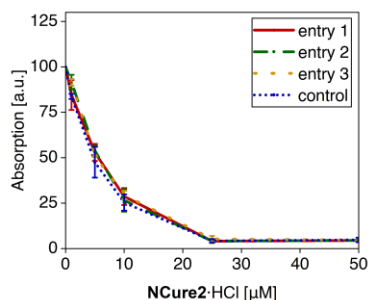


Figure 4. Effect of **NCure2**·HCl prodrugs obtained by different methods of the viability of A2780 cells.

Conclusions

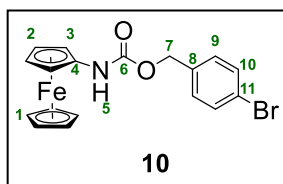
We developed an alternative synthetic route to lysosome-targeting anticancer prodrug **NCure2**·HCl. By employing high-yielding Miyaura borylation in the final step to introduce the ROS-sensitive boronic acid pinacol ester, chromatographic purification was avoided during product isolation, minimizing losses. Atomic emission spectroscopy confirmed residual amounts of palladium ($< 0.19\%$), which, as shown by MTT assays on human ovarian cancer A2780 cells, did not affect the prodrug's cytotoxicity. This improved synthesis enhances the accessibility of **NCure2**·HCl, facilitating its preclinical and eventual clinical studies. Moreover, it offers a more scalable approach. However, further optimization is required to eliminate chromatography in the first two steps and develop a more effective protocol for palladium removal.

Experimental Section

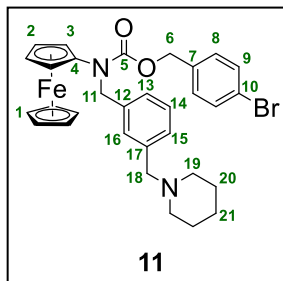
General. Chemicals were used as received, if not stated otherwise. Dichloromethane (DCM) and EtOAc were distilled from K_2CO_3 before usage. Hexanes were distilled before usage. Thin Layer Chromatography (TLC) was performed on Merck silica gel 60 F254. Compounds on TLCs were visualized under ultraviolet-light lamp (254 nm

or 366 nm). Column chromatography was performed on deactivated Macherey-Nagel silica gel 60 M (230 - 400 mesh, 0.04 - 0.063 mm). Given solvent ratios used for TLCs refer to volumes. Nuclear Magnetic Resonance (NMR) spectroscopy experiments were performed on Bruker Avance Neo 400 (^1H : 400 MHz, ^{13}C : 100 MHz), Bruker Avance Neo 500 (^1H : 500 MHz, ^{13}C : 126 MHz) or Bruker Avance Neo 600 Cryo Probe DCH (^1H : 600 MHz, ^{13}C : 150 MHz). All NMR experiments were carried out at 22 °C. Chemical shifts (δ 's) are referenced to residual proton impurities of stated solvents or to deuterated solvents itself.¹ The shortcut 'ps' refers to pseudo singlets and 'pt' refers to pseudo triplets. NMR data were processed using MestReNova 6.0.2. High resolution mass spectrometry (HRMS) was performed on a Bruker maXis 4G UHR MS/MS spectrometer or a Bruker micrOTOF II focus TOF MS spectrometer. Elemental analysis (EA) was performed in the microanalytical laboratory of the chemical institutes of the Friedrich-Alexander-University of Erlangen-Nürnberg, Erlangen, Germany. Atomic emission spectroscopy (AES) was carried out with an Agilent 4200 MP-AES instrument for the detection of Pd using an ICP standard from Bernd Kraft (1 M solution of $\text{Pd}(\text{NO}_3)_2$ in HNO_3). Human ovarian cancer cell line A2780 was purchased from Sigma Aldrich and cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% Gibco GlutaMAX and 1% penicillin/streptomycin to a confluency of 80% at 37 °C in a chamber with 5% CO_2 . The cells were harvested using trypsin/ethylenediamine- N,N,N',N' -tetraacetic acid (EDTA, 0.025%/0.01%, w/v) diluted in phosphate-buffered saline (PBS).

Synthetic procedures



Intermediate 10 A stirred mixture of ferrocenecarboxylic azide **7** (513 mg, 2.01 mmol, 1 eq), 4 bromobenzyl alcohol (**9**, 453 mg, 2.42 mmol, 1.2 eq), and toluene (HPLC grade, 1.5 mL) was heated at reflux for 3 h. The solvent was evaporated and the desired product **10** (713 mg, 1.72 mmol, 87%) was obtained pure after silica gel column chromatography (DCM = 1, \emptyset 6.5 cm x 7 cm) as an orange crystalline solid. ^1H NMR (acetone- d_6 , 400 MHz): δ [ppm] = 8.09 (s, 1H, **5**); 7.58 - 7.56 (m, 2H, **10**); 7.39 - 7.37 (m, 2H, **9**); 5.12 (s, 2H, **7**); 4.55 (ps, 2H, **3**); 4.09 (s, 5H, **1**); 3.93 (ps, 2H, **2**). $^{13}\text{C}\{^1\text{H}\}$ NMR (acetone- d_6 , 400 MHz) δ [ppm] = 154.5 (**6**); 137.6 (**8**); 132.3 (**10**); 130.7 (**9**); 122.1 (**11**); 97.4 (**4**); 69.6 (**1**); 65.8 (**7**); 64.6 (**2**); 61.0 (**3**). HRMS APPI (DCM) for $\text{C}_{18}\text{H}_{16}\text{BrFeNO}_2$, calculated: 412.9708 m/z , found: 412.9709, err [ppm] = 0.4. Elemental analysis (EA): for $\text{C}_{18}\text{H}_{16}\text{BrFeNO}_2$, calculated: C(52.2); H(3.9); N(3.4), found: C(52.3); H(3.8); N(3.5). TLC: R_f (DCM) = 0.42 - 0.51.



Intermediate 11 A mixture of intermediate **10** (99 mg, 239 μmol , 1 eq), Cs_2CO_3 (241 mg, 740 μmol , 3.06 eq), CsI (73 mg, 281 μmol , 1.17 eq), and DMF (dry, 2.2 mL) was stirred for 30 min at 22 °C under N_2 -atmosphere resulting in an orange suspension. N -((3-chloromethyl)benzyl)piperidinium chloride (**4**, 71 mg, 273 μmol , 1.14 eq) was added and the mixture was stirred at 22 °C under N_2 -atmosphere for a further 69 h. Solids were filtered off and the filtrate was evaporated. Pure product **11** (94 mg, 156 μmol , 65%) was obtained after silica gel column chromatography (DCM/ NEt_3 = 100/1, \emptyset 4 cm x 7 cm) as an orange oil. ^1H NMR (acetone- d_6 , 400 MHz, rt): δ [ppm] = 7.50 - 7.17 (m, 8H, **8+9+13-16**); 5.18 (s, 2H, **6**); 4.97 (s, 2H, **11**); 4.44 (ps, 2H, **3**); 4.11 (s, 5H, **1**); 3.97 (ps, 2H, **2**); 3.40 (s, 2H, **18**); 2.32 (ps, 4H, **19**); 1.54 - 1.48 (m, 4H, **20**); 1.41 (ps, 2H, **21**). $^{13}\text{C}\{^1\text{H}\}$ NMR (acetone- d_6 , 150 MHz, rt) δ [ppm] = 155.1 (**5**); 140.3 (**17**); 139.6 (**12**); 137.1 (**7**); 132.2; 130.8; 129.0; 128.2; 127.5; 125.7; 122.2 (**10**); 102.3 (**4**); 69.7 (**1**); 67.0 (**6**); 65.0 (**2**); 64.1 (**19**); 63.2 (**3**); 55.1 (**11**); 54.2 (**18**); 26.7 (**20**); 25.1 (**21**). HRMS APPI (DCM) for $\text{C}_{31}\text{H}_{34}\text{BrFeN}_2\text{O}_2$, calculated: 601.1148, found: 601.1148, err [ppm] = 0.3. EA for $\text{C}_{31}\text{H}_{33}\text{BrFeN}_2\text{O}_2$, calculated: C(61.9); H(5.5); N(4.7), found: C(61.7); H(5.5); N(4.6). TLC: R_f (DCM/ NEt_3 = 100/1) = 0.22 - 0.31.

Acknowledgements

We thank the German Research Council (DFG MO1418/7-2) for their financial support.

Supplementary Material

Additional spectra are provided in the supporting Information.

References

1. Cadahía, J. P.; Previtali, V.; Troelsen, N. S.; Clausen, M. H. *MedChemComm.* **2019**, *10*, 1531.
<https://doi.org/10.1039/C9MD00169G>
2. Hagen, H.; Marzenell, P.; Jentzsch, E.; Wenz, F.; Veldwijk, M. R.; Mokhir, A. *J. Med. Chem.* **2012**, *55*, 924.
<https://doi.org/10.1021/jm2014937>
3. Marzenell, P.; Hagen, H.; Sellner, L.; Zenz, T.; Grinyte, R.; Pavlov, V.; Daum, S.; Mokhir, A. *J. Med. Chem.* **2013**, *56*, 6935.
<https://doi.org/10.1021/jm400754c>
4. Daum, S.; Chekhun, V. F.; Todor, I. N.; Lukianova, N. Y.; Shvets, Y. V.; Sellner, L.; Putzker, K.; Lewis, J.; Zenz, T.; de Graaf, I. A. M.; Groothuis, G. M. M.; Casini, A.; Zozulia, O.; Hampel, F.; Mokhir, A. *J. Med. Chem.* **2015**, *58*, 2015.
<https://doi.org/10.1021/jm5019548>
5. Jaouen, G.; Vessieres, A.; Top, S. *Chem. Soc. Rev.* **2015**, *44*, 8802.
<https://doi.org/10.1039/C5CS00486A>
6. Maslah, H.; Skarbek, C.; Pethe, S.; Labruere, R. *Eur. J. Med. Chem.* **2020**, *207*, 112670.
<https://doi.org/10.1016/j.ejmech.2020.112670>
7. Daum, S.; Reshetnikov, M. S. V.; Sisa, M.; Dumych, T.; Lootsik, M. D.; Bilyy, R.; Bila, E.; Janko, C.; Alexiou, C.; Herrmann, M.; Sellner, L.; Mokhir, A. *Angew. Chem. Int. Ed.* **2017**, *56*, 15545.
<https://doi.org/10.1002/anie.201706585>
8. Endo, K.; Kurosawa, F.; Ukay, Y. *Chem. Lett.* **2013**, *42*, 1363.
<https://doi.org/10.1246/cl.130643>
9. He, L.-Y.; Schulz-Senft, M.; Thiedemann, B.; Linshoef, J.; Gates, P. J.; Staubitz, A. *Eur. J. Org. Chem.* **2015**, 2498.
<https://doi.org/10.1002/ejoc.201500138>
10. Oka, N.; Yamada, T.; Sajiki, H.; Akai, S.; Ikawa, T. *Org. Lett.* **2022**, *24*, 3510.
<https://doi.org/10.1021/acs.orglett.2c01174>
11. Goggins, S.; Apsey, E. A.; Mahon, M. F.; Frost, C. G. *Org. Biomol. Chem.* **2017**, *15*, 2459.
<https://doi.org/10.1039/C7OB00211D>
12. Gao, F.; Wang, F.; Nie, X.; Zhang, Z.; Chen, G.; Xia, L.; Wang, L.-H.; Wang, C.-H.; Hao, Z.-Y.; Zhang, W.-J.; Hong, C.-Y.; You, Y.-Z. *New. J. Chem.* **2020**, *44*, 3478.
<https://doi.org/10.1039/C9NJ05860E>
13. Özkan, H. G.; Thakor, V.; Xu, H.-G.; Bila, G.; Bilyy, R.; Bida, D.; Böttcher, M.; Mougiakakos, D.; Tietze, R.; Mokhir, A. *Chem. Eur. J.* **2022**, *28*, e202104420.

14. <https://doi.org/10.1002/chem.202104420>
Liu, T. Z.; Lee, S. D.; Bhatnagar, R. S. *Toxicol. Lett.* **1979**, *4*, 469.
[https://doi.org/10.1016/0378-4274\(79\)90113-9](https://doi.org/10.1016/0378-4274(79)90113-9)
15. Kielhorn, J.; Melber, C.; Keller, D.; Mangelsdorf, I. *Int. J. Hyg. Heal.* **2002**, *205*, 417.
<https://doi.org/10.1078/1438-4639-00180>
16. Leso, V.; Iavicoli, I. *Int. J. Mol. Sci.* **2018**, *19*, 503.
<https://doi.org/10.3390/ijms19020503>

This paper is an open access article distributed under the terms of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>)