Synthesis of a lysosome-targeting aminoferrocene-based prodrug NCure2

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Abstract

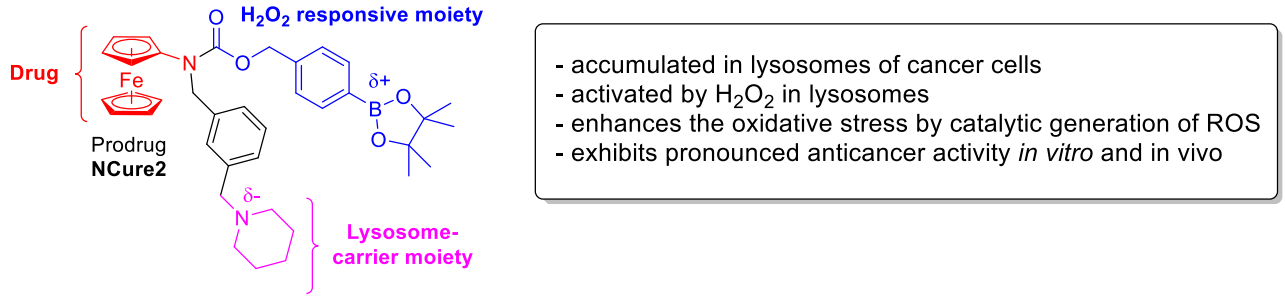
Prodrugs can achieve highly specific targeting of cancer cells. Therefore, their application can improve the therapy of cancer diseases. The best aminoferrocene-based prodrug developed in the group of Mokhir is N-ferrocenyl-N-[4-(1-piperidinylmethyl)benzyl]aminocarboyloxymethyl-phenylboronic acid pinacol ester, **NCure2** (*Angew. Chem. Int. Ed.* 56 (2017), 15545). It targets lysosomes of cancer cells due to the presence of a basic *N*-alkylpiperidine moiety in its structure. In the lysosomes, the prodrug is activated by H_2O_2 due to the presence of a H_2O_2-responsive, Lewis acidic boronic acid pinacol ester moiety. Due to the zwitter-ionic nature of **NCure2** its synthesis is low yielding, purification is laborious that leads to the product losses. In this work we report on the 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 allowed the facile purification of the final product by its precipitation. This new approach makes **NCure2**·HCl easily synthetically accessible that will facilitate its further pre-clinical and clinical studies.

Introduction

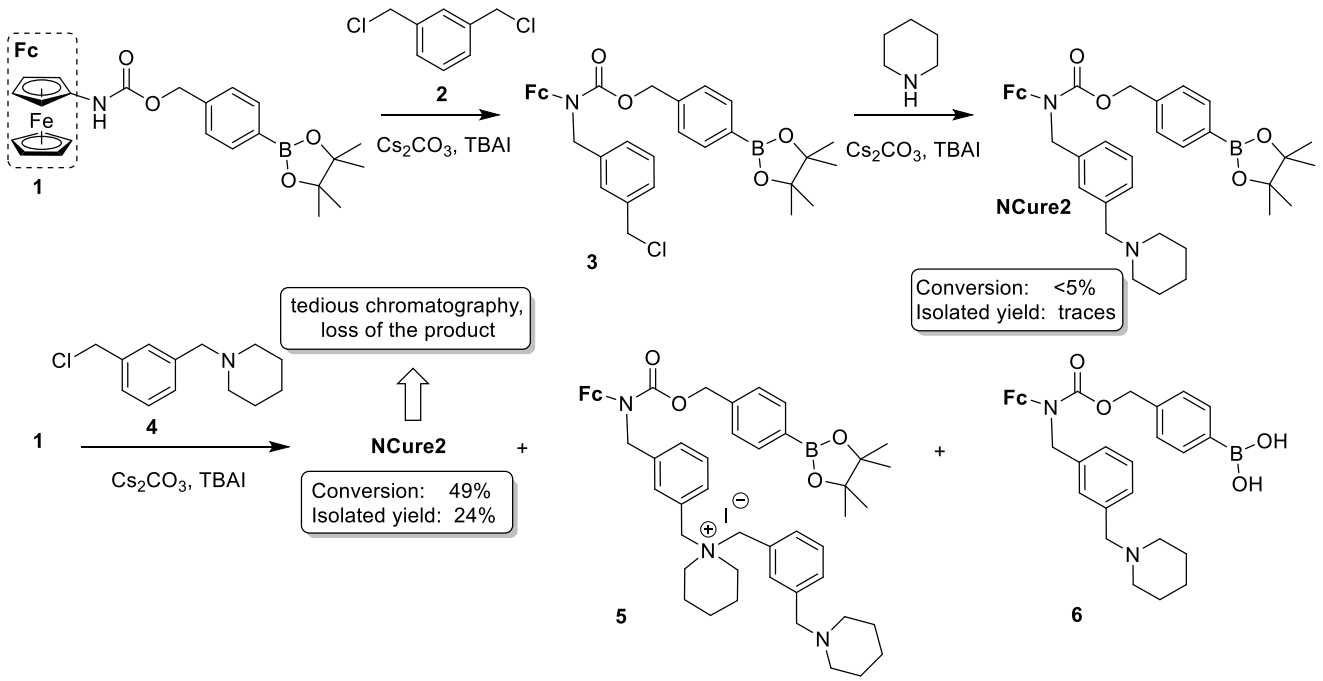
One possible approach for precise targeting of cancer cells is based on the prodrug concept. A prodrug is an inactive compound, which can be activated in cancer specific environment with formation of an active drug.[1] Since healthy cells do not possess such an environment, the prodrug remains inactive in these cells. For example, the group of Mokhir as well as other research groups have explored differences in the level of H_2O_2 in cancer and healthy cells.[2-6] H_2O_2 is the most abundant intracellular reactive oxygen species (ROS). One of the most successful H_2O_2-responsive prodrugs activated by H_2O_2, which has been developed in the group of Mokhir up to date, is N-ferrocenyl-N-[4-(1-piperidinylmethyl)benzyl]aminocarboyloxymethyl-phenylboronic acid pinacol ester, called **NCure2**.[7] Its chemical structure is shown in Scheme 1. **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 H_2O_2 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. At the same time, **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 Cs_2CO_3, 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 traces of the desired product only due to the presence of the large number of not fully identified 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 not identified products are also formed. Some of these side products are difficult to separate from **NCure2** that reduces the yield of the isolated product down to 24%. The latter optimized protocol was extensively used by our group for the preparation of 10 – 100 mg of **NCure2** in one run that is sufficient for the majority of *in vitro* and preliminary *in vivo* studies. However, for the further pre-clinical evaluation of this prodrug, e.g. *in vivo* studies in different cancer models in mice and rats, its large quantities (>100 mg) are required. The current work addresses this need by reporting the improved protocol allowing the facile scale-up 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.



State-of-the-art: known methods of synthesis of prodrug **NCure2**



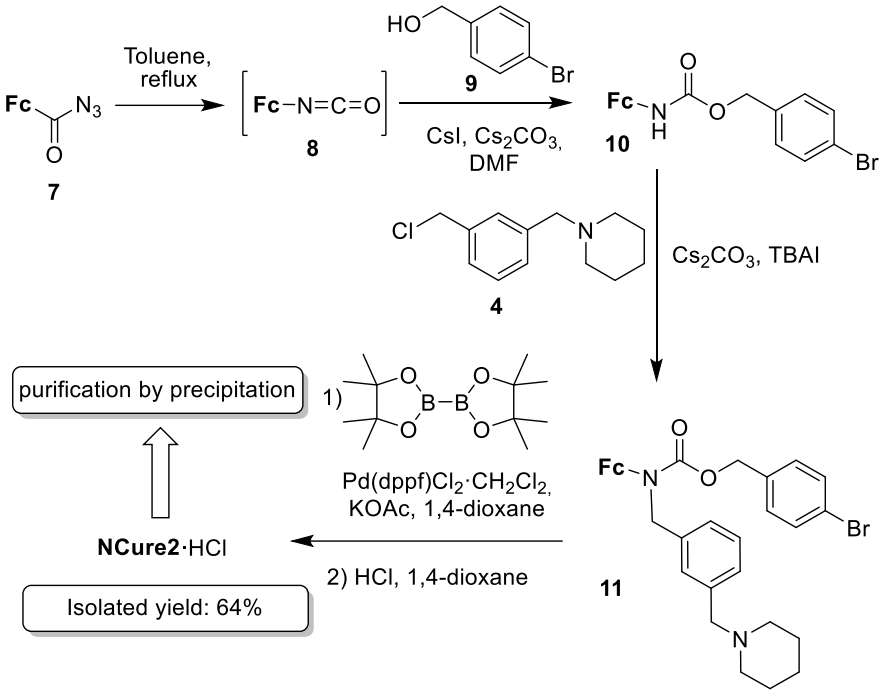
Scheme 1. A structure of prodrug **NCure2** highlighting with different colors the moieties important for its activity and outlining some of its properties. Known approaches for synthesis of the **NCure2** are summarized in the "state-of-the-art" section.[7]

Results and discussion

NCure2 is a zwitterionic 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, since it runs on the column as a broad band under all neutral, basic or acidic conditions. 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 account for these challenges, we planned the synthesis of **NCure2** in a

way that the last step is high yielding and producing side products, which can be removed by non-chromatographic methods. The synthetic approach is outlined in Scheme 2.

This work: improved synthesis of prodrug **NCure2**



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

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 1-bromo-4-(ferrocenylaminocarbonyloxamethyl)benzene (**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**. **11** was purified by silica column chromatography using the trimethylamine-containing eluent mixture to keep the aliphatic amine moiety in **11** in the deprotonated state thereby avoiding tailing of the band of this compound. The purification under these conditions was trivial and yielded 65% of **11**. In the final step, the borate ester was introduced *via* Miyaura borylation towards **NCure2**. The product mixture was filtered through Celite® with charcoal topping, which removes palladium residues. Afterwards, the product **NCure2** was transformed into its HCl salt by addition of a HCl solution in 1,4-dioxane. The solution was filtered once more to get rid of potential 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) (found *m/z* 648.2824 Da, calcd. for $C_{37}H_{45}BFeN_2O_4$ 648.2816, error = -0.1 ppm) as well as ^1H and ^{13}C NMR spectroscopy (Figures S5 & S6, supporting information (*SI*)). The purity of the product was confirmed by thin layer chromatography in different eluents (retention factor (R_f) in CH_2Cl_2 / NEt_3 = 100/1, v/v = 0.17 ± 0.02; R_f in EtOAc = 0.23 ± 0.05; R_f in EtOAc / NEt_3 = 100/1, v/v = 0.72 ± 0.07) and elemental analysis (found: carbon, 63.3%; hydrogen, 6.7%; nitrogen, 3.9%; calcd. for $C_{37}H_{46}BClFeN_2O_2 \cdot H_2O$: carbon, 63.2%; hydrogen, 6.9% and nitrogen, 4.0%).

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 ^{1}H NMR spectroscopy, both samples were found to be practically identical (Figure 1).

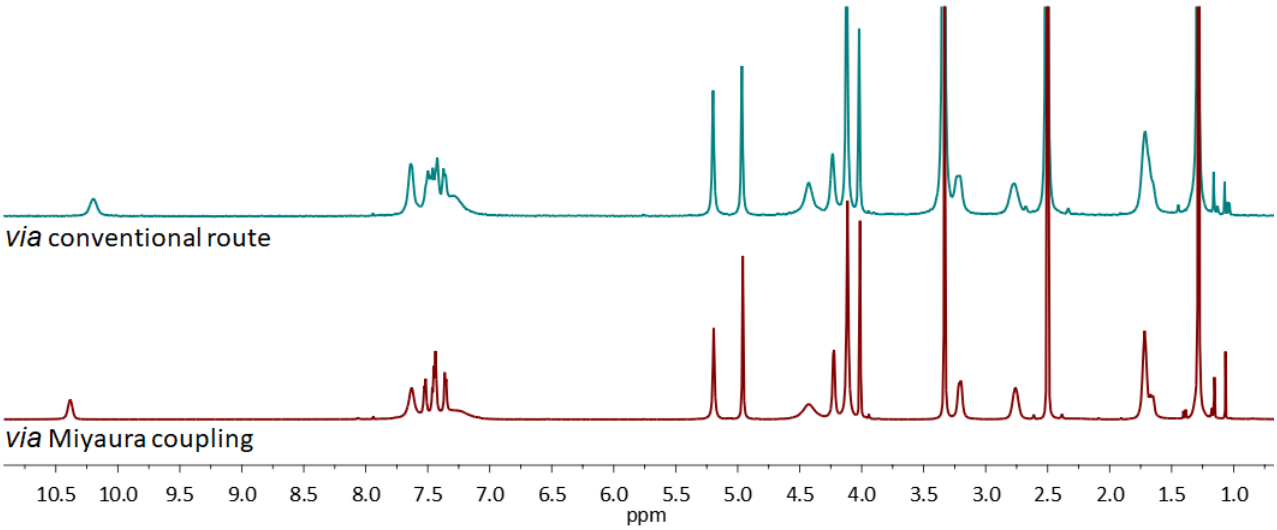


Figure 1. ^{1}H NMR spectra of **NCure2**·HCl synthesized by the conventional route (Angew. Chem. 2017) (upper trace) and Miyaura borylation (lower trace).

We obtained 453 mg of analytically pure **NCure2**·HCl in a single run without the need for tedious chromatographic purification. 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 μg/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 2A).

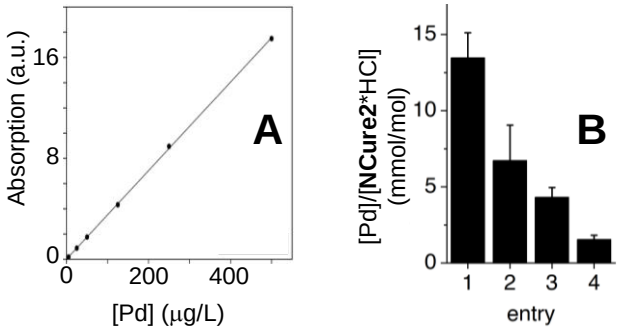


Figure 2. Atomic emission spectroscopy (AES) data. **A**: A calibration plot of the AES response (OY axis: absorbance, expressed in arbitrary units, a.u.) *versus* the concentration of $Pd(NO_3)_2$ solution in HNO_3 (OX axis: [Pd]). **B**: Content of Pd in samples of **NCure2**·HCl prepared by the new 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 2B). 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: $p<0.01$. However, the absolute palladium amount is small: 0.04 – 0.19% Pd in the samples of the prodrug.

To exclude that the Pd traces will have some effect of the biological activity of the **NCure2**·HCl, we have 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 3). 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 ± 0.02 μM to 5.62 ± 0.50 μM, entries 1-3) are the same as the IC_{50} for the **NCure2**·HCl obtained by the conventional method (4.97 ± 1.13 μM, 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.

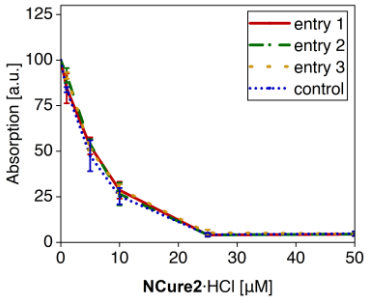


Figure 3. Effect of **NCure2**·HCl prodrugs obtained by different methods of the viability of A2780 cells.

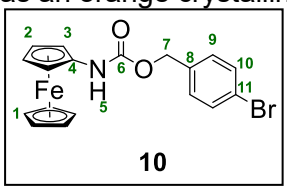
Experimental

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. Nuclei Magnetic Resonance (NMR) spectroscopy experiments were performed on Bruker Avance Neo 400 (^{1}H: 400 MHz, ^{13}C: 100 MHz), Bruker Avance Neu 500 (^{1}H: 500 MHz, ^{13}C: 126 MHz) or Bruker Avance Neo 600 Cryo Probe DCH (^{1}H: 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] NMR data were processed using MestReNova 6.0.2. High resolution mass spectrometry (HRMS) was performed on mass spectrometer 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 the Agilent 4200 MP-AES for the detection of Pd using an ICP standard from Bernd Kraft (1 M solution of $Pd(NO_3)_2$ 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).

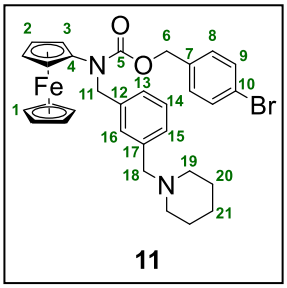
Synthesis

Intermediate **10** Ferrocenecarboxylic azide (**7**, 513 mg, 2.01 mmol, 1 eq) was put together with 4-bromobenzyl alcohol **9** (453 mg, 2.42 mmol, 1.2 eq) and a stir bar into a 5 mL pear-shaped flask. Toluene (HPLC grade, 1.5 mL) was added and the mixture was heated to reflux. The mixture was kept at the latter conditions 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, Ø 6.5 cm x 7 cm) as an orange crystalline solid. ^{1}H NMR (acetone-d6, 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}C{^{1}H} NMR (acetone-d6, 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 $C_{18}H_{16}BrFeNO_2$, calculated: 412.9708 *m/z*, found: 412.9709, err [ppm] = 0.4. Elemental analysis (EA): for $C_{18}H_{16}BrFeNO_2$, 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.



10

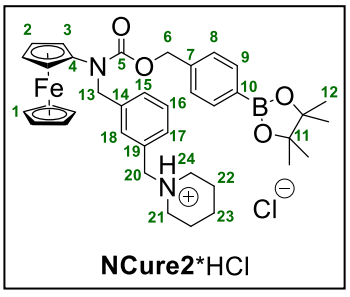
Intermediate **11** Intermediate **10** (99 mg, 239 µmol, 1 eq) was put together with Cs_2CO_3 (241 mg, 740 µmol, 3.06 eq), CsI (73 mg, 281 µmol, 1.17 eq) and a stir bar into a 5 mL Schlenk tube. DMF (dry, 2.2 mL) was added under N_2-atmosphere resulting in an orange suspension. The mixture was stirred for 30 min under N_2-atmosphere at 22 °C and *N*-((3-chloromethyl)benzyl)piperidinium chloride **4** (71 mg, 273 µmol, 1.14 eq) was added. Then, the mixture was stirred at 22 °C under N_2-atmosphere for another 69 h. Solids were filtered off and the solvent was evaporated. Pure product **11** (94 mg, 156 µmol, 65%) was obtained after silica gel column chromatography (DCM/NEt_3 = 100/1, Ø 4 cm x 7 cm) as an orange oil. ^{1}H NMR (acetone-d6, 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}C{^{1}H} NMR (acetone-d6, 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 $C_{31}H_{34}BrFeN_2O_2$, calculated: 601.1148, found: 601.1148, err [ppm] = 0.3. EA for $C_{31}H_{33}BrFeN_2O_2$, 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.



11

Prodrug **NCure2**·HCl Intermediate **11** (610 mg, 1.01 mmol, 1 eq) was put together with $(BPin)_2$ (293 mg, 1.15 mmol, 1.14 eq), KOAc (298 mg, 3.04 mmol, 3 eq), $Pd(dppf)Cl_2 \cdot CH_2Cl_2$ (97 mg, 119 µmol, 0.12 eq) and a stir bar into a 5 mL microwave tube. 1,4-Dixoane (3.4 mL) was added and the mixture was degassed with N_2 for 5 min. The tube was sealed and heated to 80 °C in an oil bath for 19 h. The mixture was allowed to cool to 22 °C. The crude was filtered via Celite© plug filtration with charcoal topping (EtOAc = 1, Ø 3 cm x 2 cm) to remove impurities and Pd residues.

HCl salt formation: The product was dissolved in EtOAc (25 mL) and a solution of HCl in 1,4-dioxane (4 M, dry, 10 mL, 40 eq) was added dropwise under stirring. Volatiles were removed and the mixture was dried for 20 min. Then, *iso*-propanol (20 mL) was added and the mixture was stirred vigorously for 45 min, which led to product precipitation after 20 minutes. The product **NCure2**·HCl (453 mg, 661 µmol, 64%) was obtained pure after filtration and drying for several days under vacuum. ^{1}H NMR (DMSO-d6, 400 MHz, rt): δ [ppm] = 10.02 (s, 1H, 24); 7.63 – 7.28 (m, 8H, 8+9+15-18); 5.19 (s, 2H, 6); 4.97 (s, 2H, 13); 4.43 (ps, 2H, 3); 4.24 (d, ^{2}J = 4.9 Hz, 20); 4.88 (s, 5H, 1); 4.02 (pt, ^{3}J = 1.9 Hz, 2); 3.24 – 3.21 (m, 2H, 21); 2.81 – 2.74 (m, 2H, 21); 1.75 – 1.65 (m, 5H, 22+23); 1.28 (s, 13H, 23+12). HRMS APPI (DCM) for $C_{37}H_{45}BFeN_2O_4$, calculated: 648.2816, found: 648.2824, err [ppm] = -



NCure2*HCl

0.1. EA for $C_{37}H_{46}BClFeN_2O_2 \cdot H_2O$, calculated: C(63.2); H(6.9); N(4.0), found: C(63.3); H(6.7); N(3.9). TLC: R_f (DCM/NEt_3 = 100/1) = 0.15 – 0.18.

Atomic emission spectroscopy (AES)

For AES measurements, a small amount of compound (0.3 mg – 2 mg) was suspended in aqueous HNO_3 (65%, 50 µL) and boiled for 30 min at 95 °C. Then, water (550 µL) was added and the resulting solution (30 µL) was diluted to 1 mL with water. Measurements were carried out with this diluted solution and measured intensities were converted into concentrations via the calibration curve (Figure 2A).

Experiments with A2780 cells

A2780 cells were resuspended in RPMI 1640 medium containing 5% FBS and seeded on a 96 microtiter plate (250 cells/µL, 100 µL/well). After overnight incubation, investigated compounds dissolved in dimethyl sulfoxide (DMSO) were added (1 µL) to final concentrations of 50 µM, 25 µM, 10 µM, 5 µM and 1 µM. Each condition was tested in triplicates, while DMSO was tested in nine technical replicates. Treated cells were incubated for 48 h, after which solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/mL in PBS, 20 µL/well) was added. After further 3 h of incubation, sodium dodecyl sulfate solution (SDS, 10% in 0.01 M HCl, 90 µL/well) was added. Next day, the absorbance was measured at 690 nm and 590 nm. While 590 nm absorbance was exploited to detect intensity of metabolized MTT, absorbance at 690 nm served as baseline value (A(590 nm) – A(690 nm)). Each experiment was repeated two times. An unpaired Student`s t test was used to compare data pairs with each other. The values were considered statistically significantly different for $p<0.05$.

Conclusions

We developed an alternative synthetic route towards lysosome-targeting anticancer prodrug **NCure2**·HCl by utilizing the high yielding Miyaura borylation for introducing the ROS-sensitive boronic acid pinacol ester in the last step. The product isolation does not require silica gel chromatography that allowed minimization of the product loss. The reported here synthesis is suitable for scaling up the preparation of **NCure2**·HCl. Atomic emission spectroscopy confirmed the presence of minor Pd concentrations in the final products (<0.19 % Pd). However, these Pd traces do not affect the cytotoxicity of the prodrugs as confirmed by the MTT assays on human ovarian cancer A2780 cells. The easy synthetic accessibility of **NCure2**·HCl will foster its pre-clinical and eventually clinical studies.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

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Supporting Information

Additional Spectra are provided in the corresponding supporting Information.

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Synthesis of a lysosome-targeting aminoferrocene-based prodrug NCure2 - Supporting Information

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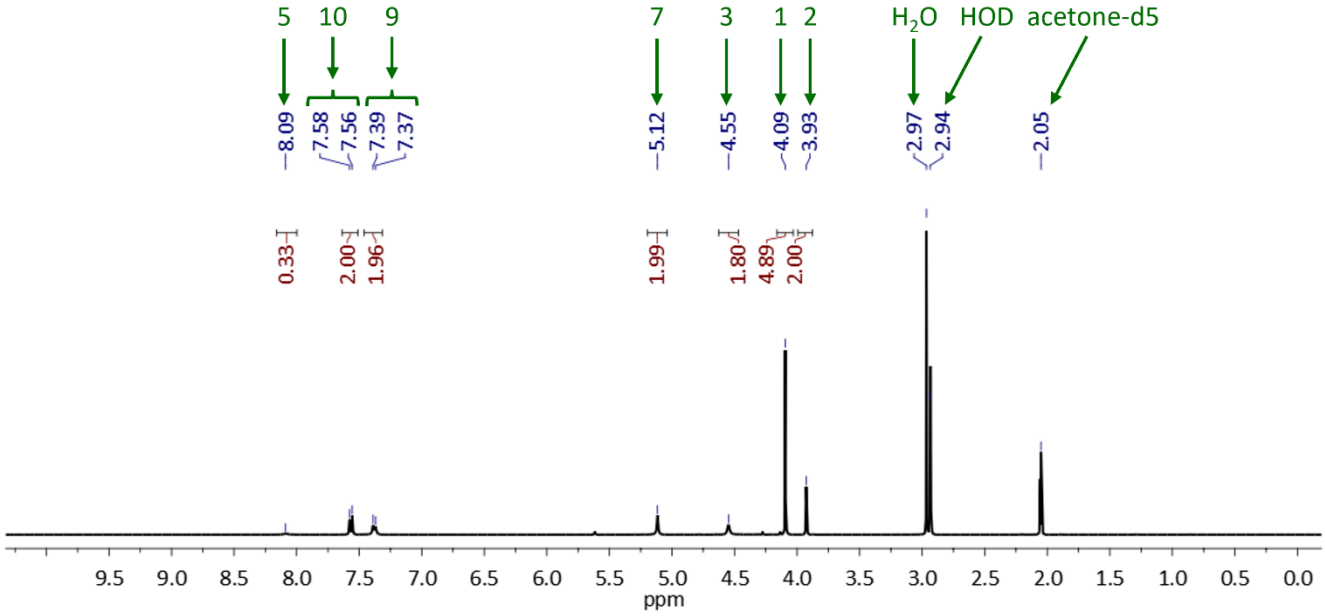
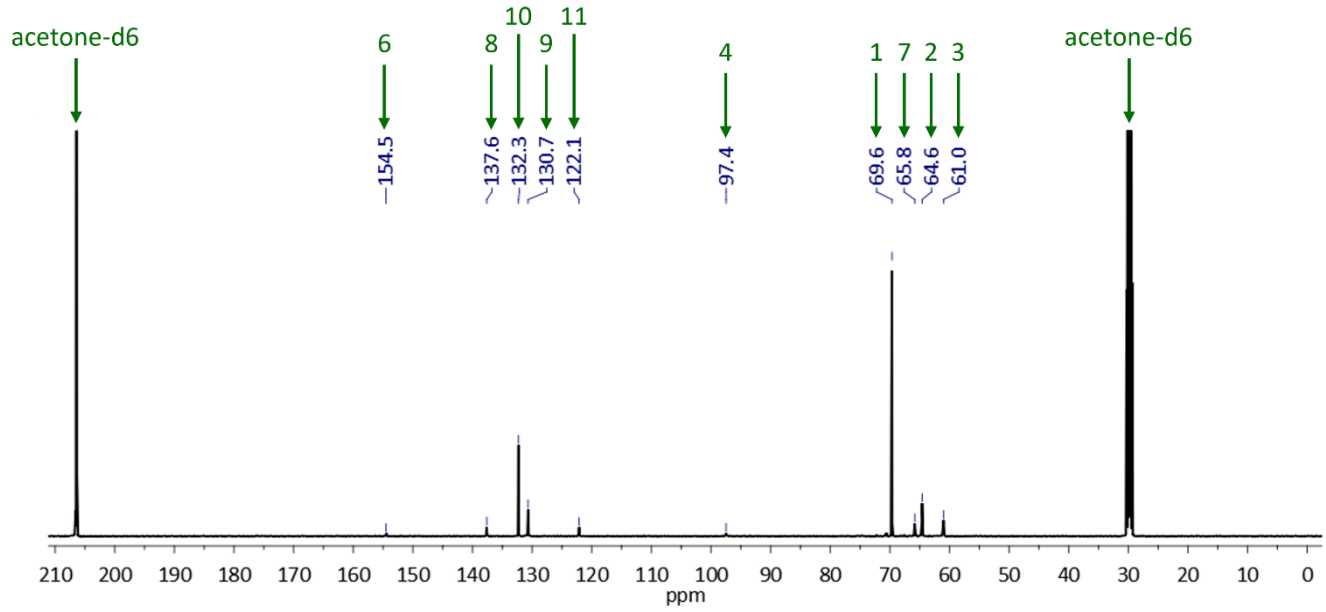
Content

1. NMR Spectra

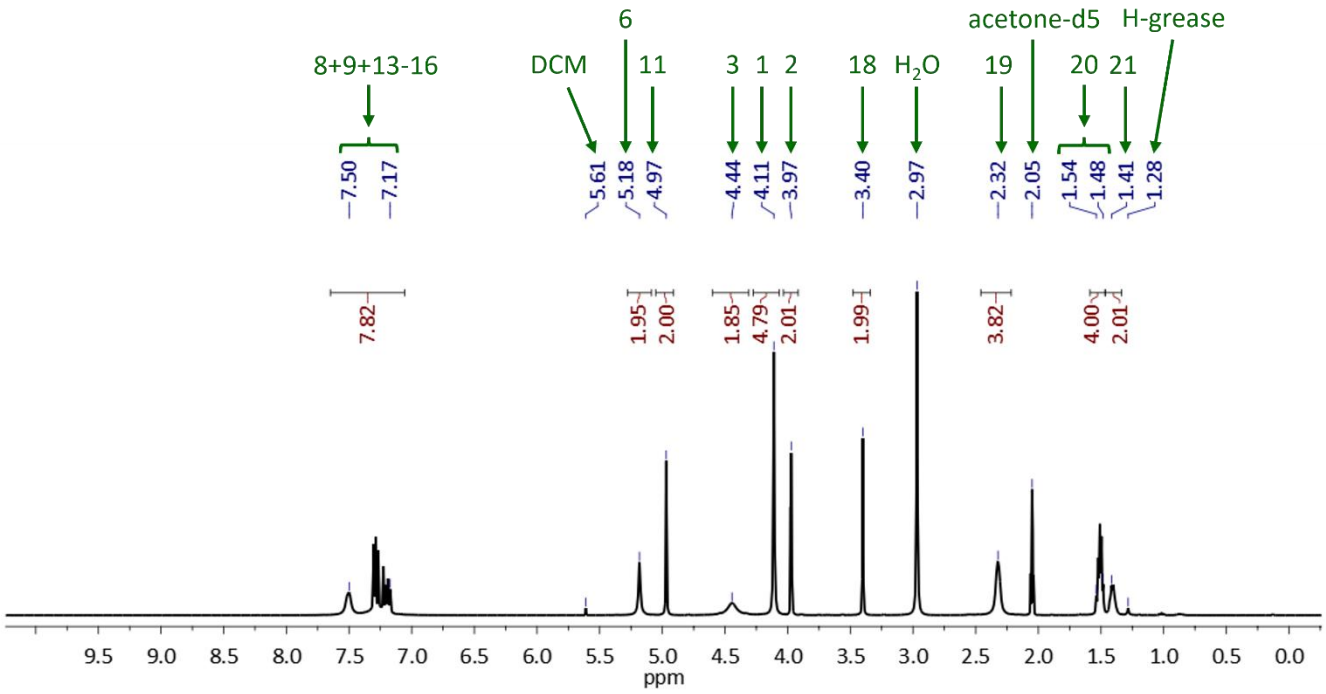
^{1}H NMR of 4-bromobenzyl ferrocylcarbamate (**10**, acetone-d6, 400 MHz):



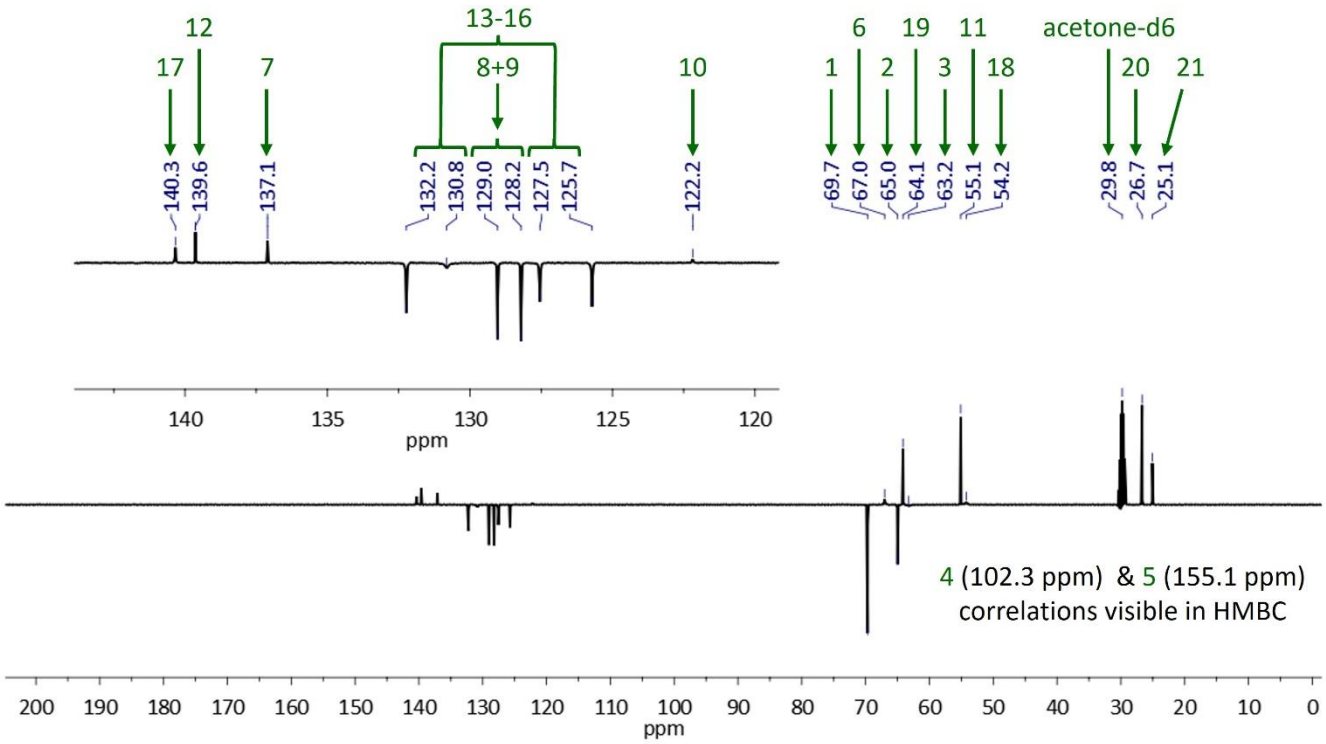
^{13}C{^{1}H} NMR of 4-bromobenzyl ferrocylcarbamate (**10**, acetone-d6, 126 MHz):



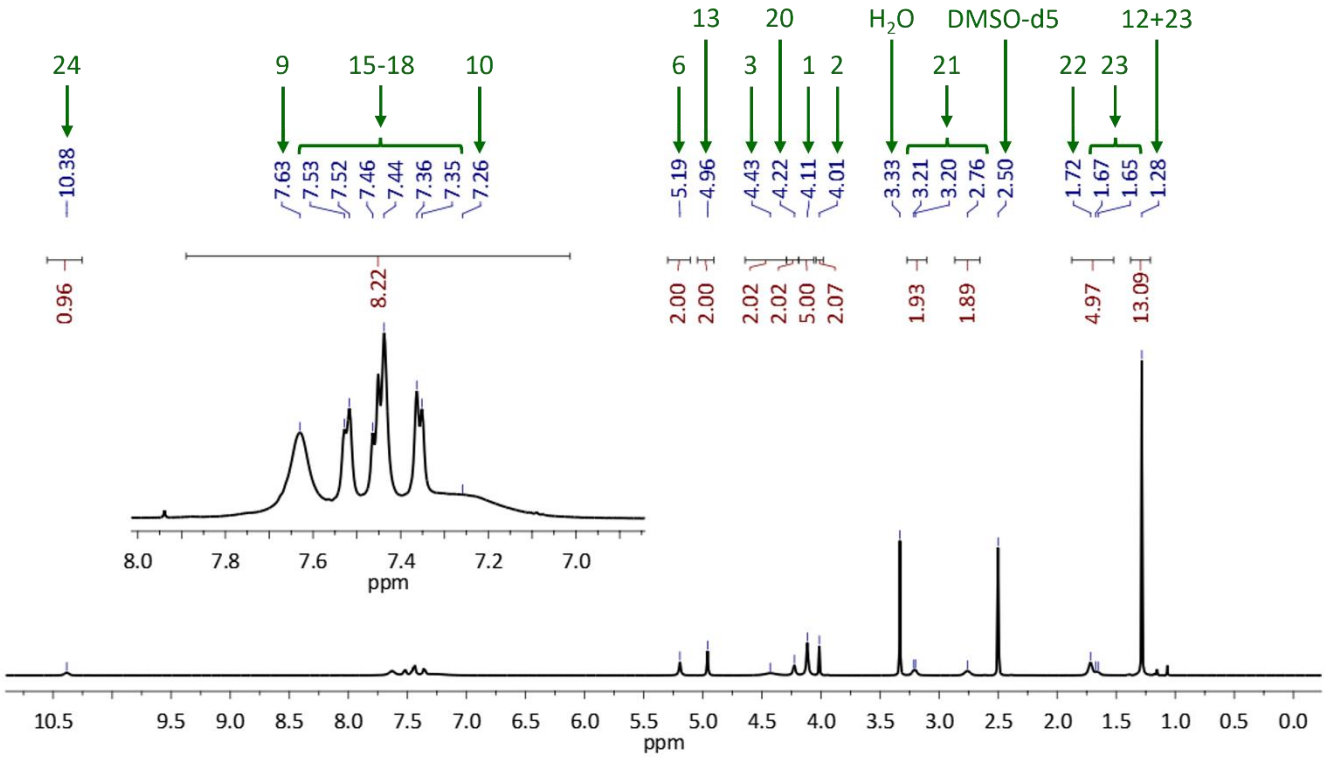
^{1}H NMR of 4-bromobenzyl ferrocyl(misyl)carbamate (**11**, acetone-d6, 400 MHz):



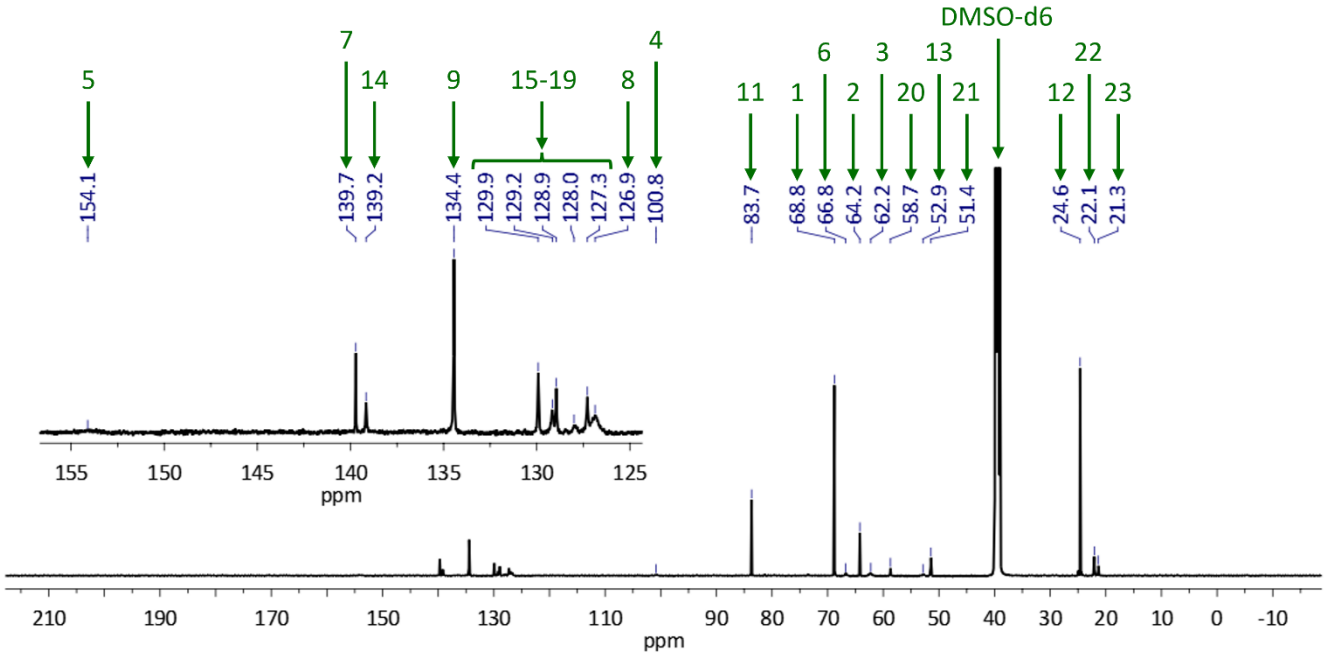
^{13}C{^{1}H} DEPTQ NMR of 4-bromobenzyl ferrocylcarbamate (**11**, acetone-d6, 100 MHz):



^{1}H NMR of 4-(pinacolboranyl)benzylferrocyl(misyl)carbamate hydrochloride (**NCure2**·HCl, DMSO-d6, 600 MHz):



^{13}C{^{1}H} NMR of 4-(pinacolboranyl)benzylferrocyl(misyl)carbamate hydrochloride (**NCure2**·HCl, DMSO-d6, 150 MHz):

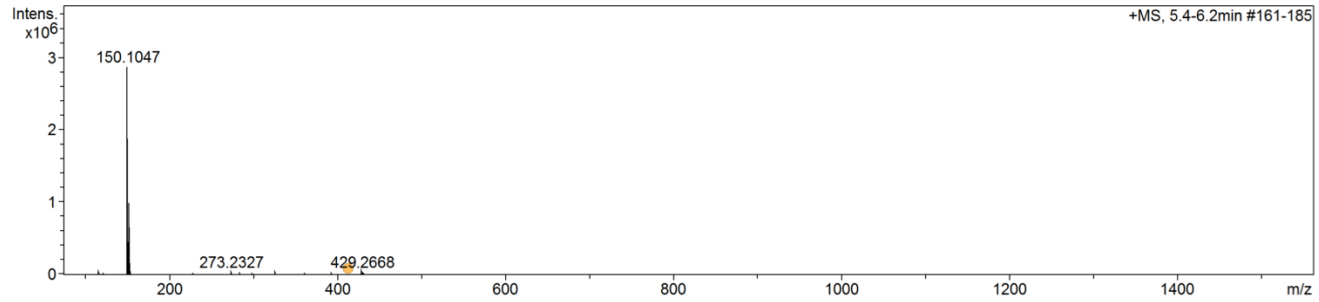


2. HRMS Spectra

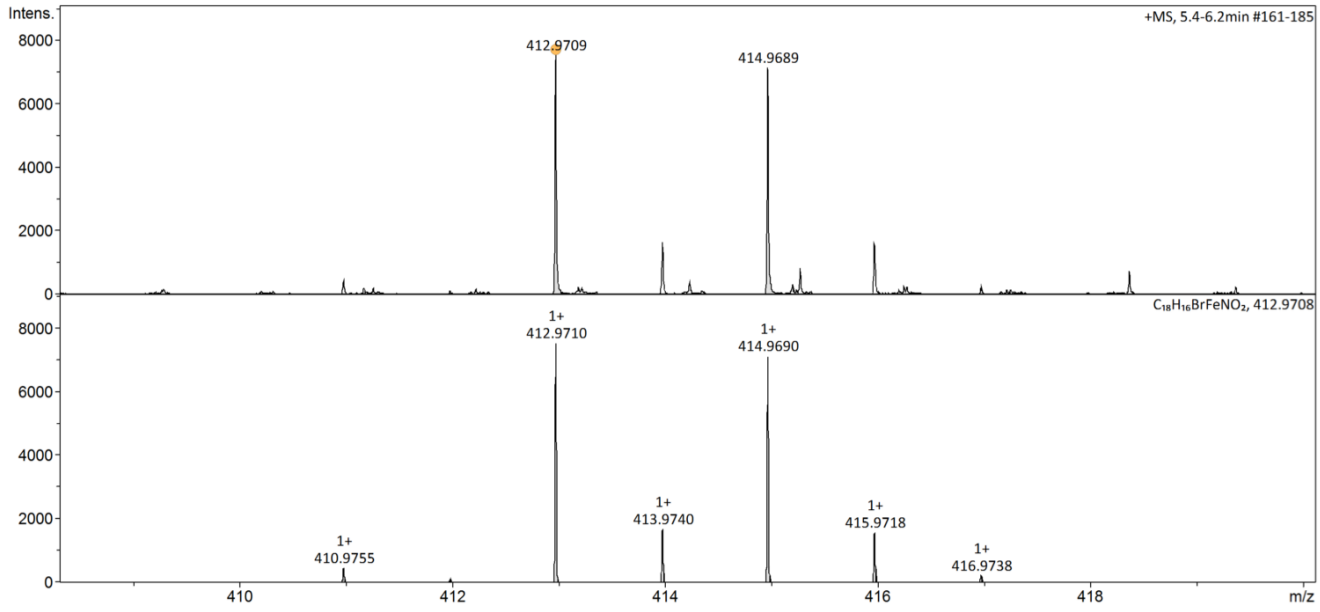
HRMS of 4-bromobenzyl ferrocylcarbamate (**10**):

Acquisition Parameter

Source Type	APPI	Ion Polarity	Positive	Set Nebulizer	5.2 Bar
Focus	Not active	Set Capillary	700 V	Set Dry Heater	220 °C
Scan Begin	80 m/z	Set End Plate Offset	-500 V	Set Dry Gas	1.2 l/min
Scan End	1550 m/z	Set Charging Voltage	0 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	250 °C



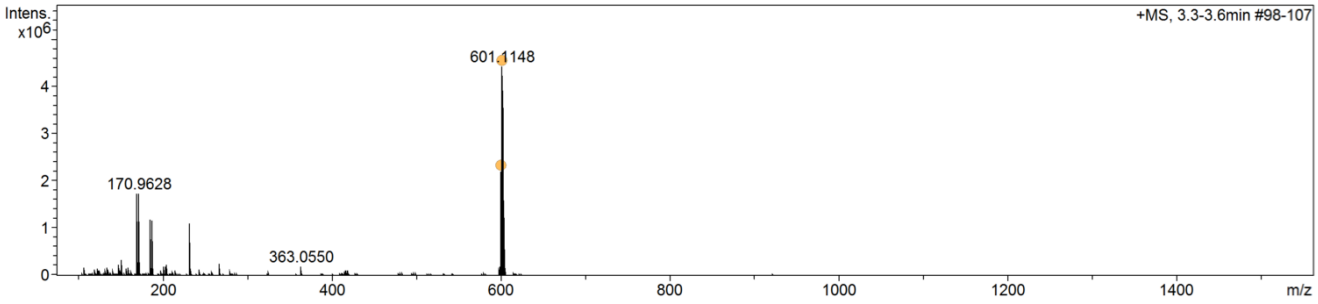
Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
412.9709	1	C18H16BrFeNO2	412.9708	0.4	117.9	1	100.00	11.0	odd	ok



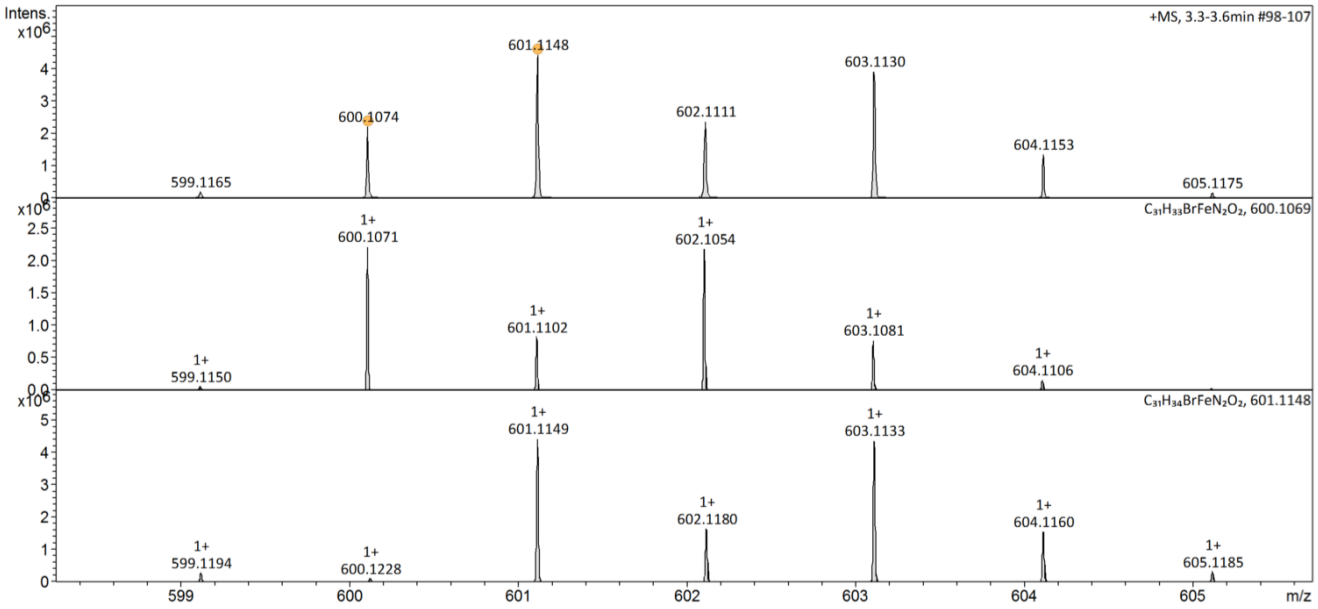
HRMS of 4-bromobenzyl ferrocyl(misyl)carbamate (**11**):

Acquisition Parameter

Source Type	APPI	Ion Polarity	Positive	Set Nebulizer	5.2 Bar
Focus	Not active	Set Capillary	700 V	Set Dry Heater	220 °C
Scan Begin	80 m/z	Set End Plate Offset	-500 V	Set Dry Gas	1.2 l/min
Scan End	1550 m/z	Set Charging Voltage	0 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	250 °C



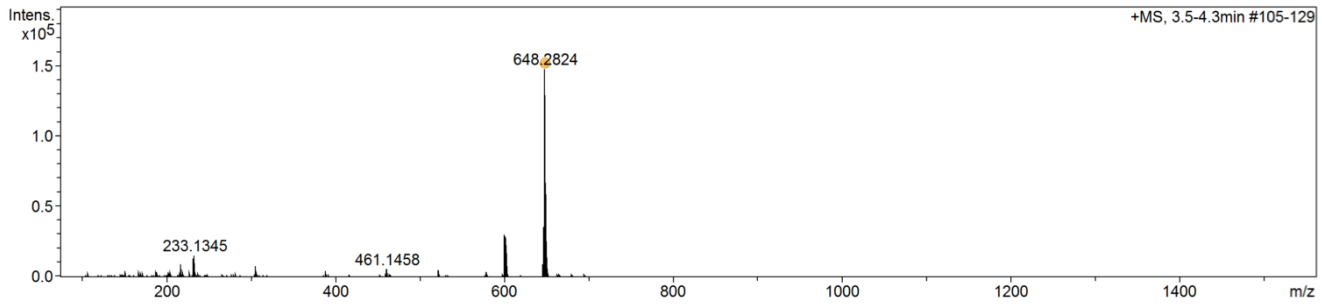
Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
600.1074	1	C31H33BrFeN2O2	600.1069	-0.5	413.9	1	100.00	16.0	odd	ok
601.1148	1	C31H34BrFeN2O2	601.1148	0.3	76.9	1	100.00	15.5	even	ok



HRMS of 4-(pinacolboranyl) ferrocyl(misyl)carbamate hydrochloride salt (**NCure2**·HCl):

Acquisition Parameter

Source Type	APPI	Ion Polarity	Positive	Set Nebulizer	5.2 Bar
Focus	Not active	Set Capillary	700 V	Set Dry Heater	220 °C
Scan Begin	80 m/z	Set End Plate Offset	-500 V	Set Dry Gas	1.2 l/min
Scan End	1550 m/z	Set Charging Voltage	0 V	Set Divert Valve	Waste
		Set Corona	0 nA	Set APCI Heater	250 °C



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
648.2824	1	C37H45BFeN2O4	648.2816	-0.1	15.1	1	100.00	17.0	odd	ok