Supplementary Information

A successful search for new, efficient, and silver-free manufacturing processes for key platinum(II) intermediates applied in antibody-drug conjugate (ADC) production

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7. References

1. Materials and methods

1.1. General considerations

All reagents were used as purchased without further purification, unless otherwise stated.

Dichlorido(ethylenediamine)platinum(II) (**2a**, Pt(ethane-1,2-diamine)Cl_2, $PtCl_2$(en) or *Lx*Cl_2), was obtained from Sigma-Aldrich (99%, [14096-51-6], product code 244929) and was used as obtained without further purification.

Potassium tetrachloroplatinate(II) (99+%, [10025-99-7], product code 053555) was obtained from Fluorochem and was used as obtained without further purification. Potassium iodide was obtained from Sigma-Aldrich (99.5%, [7681-11-0], product code 30315) and was used as obtained without further purification. Sodium bromide was obtained from Baker (99%, [7647-15-6], chlorine content: 0.15%) and was used as obtained without further purification. Silver nitrate was obtained from Sigma-Aldrich (99+%, [7761-88-8], product code 209139) and was used as obtained without further purification.

Noreleagnine (1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole, **1a**) was obtained from Sigma-Aldrich (98%, [16502-01-5], product code 300764) and was used as obtained without further purification. The HPLC analysis indicated that its purity was 100% at 273 nm.

tert-Butyl 4-(aminomethyl)piperidine-1-carboxylate (**4**) was obtained from Fluorochem (97%, [144222-22-0], product code 011389) and was used as obtained without further purification.

Dry solvents used for the solvent screening experiment (section 3.3.) and for the standard complexation reactions were purchased from Sigma-Aldrich or Acros and were supplied in sealed brown bottles over MS: DMF (Acros; product code: 348430010), DMA (Sigma-Aldrich; product code: 396351000), MeOH (Acros; product code: 364390010), EtOH (Acros; product code: 397690010), MeCN (Acros; product code: 364310010), DMSO (Acros; product code: 348441000), acetone (Acros, product code: 326800010), ethyl acetate (Acros; product code: 364350010), 1-methyl-2-pyrrolidinone (NMP; Acros; product code: 326931000), 1,3-dimethyl-2-imidazolidinone (DMI; Sigma-Aldrich; product code: 40725), 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU; Sigma-Aldrich; product code: 41661).

Following solvents (DMF analogues) used for the solvent screening experiment (section 3.4.) were purchased from Sigma-Aldrich or Fluorochem and were used as obtained: *N*-formyl pyrrolidine (Sigma-Aldrich; product code: 166391), *N*-formyl piperidine (Fluorochem; product code: 208647), *N*-formyl morpholine (Sigma-Aldrich; product code: 250376), diethyl formamide (Fluorochem; product code: 049814), diisopropyl formamide (Sigma-Aldrich; product code: 065353), methyl formamide (Sigma-Aldrich; product code: 473936), formamide (Baker Chemicals B.V.; product code: 7042), tetramethyl urea (Fluorochem; product code: 094805).

Following organic bases used for the base screening experiment (section 3.5.) were purchased from Sigma-Aldrich or Fluorochem and were used as obtained: triethylamine (TEA; Sigma-Aldrich; product code: T0886), *N*,*N*-diisopropylethylamine (DIPEA; Sigma-Aldrich; product code: 496219), *N*-methyl piperidine (Fluorochem; product code: 494121), *N*-methyl morpholine (Sigma-Aldrich; product code: M56557), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU; Sigma-Aldrich; product code: 139009), 1,4-diazabicyclo[2.2.2]octane (DABCO;

Sigma-Aldrich; product code: D27802), 2,6-lutidine (Sigma-Aldrich; product code: L0255), 2,4,6-collidine (Sigma-Aldrich; product code: 142387).

High-resolution mass spectra were recorded on an Agilent mass spectrometer using ESI-TOF (electrospray ionization-time of flight).

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 250 MHz, a Bruker MSL 400 MHz or a Bruker 500 MHz spectrometer. Chemical shifts (δ) are given in ppm and were internally referenced to residual solvent signal (for $CDCl_3$: ^{1}H: δ = 7.29 ppm, ^{13}C: δ = 77.0 ppm). Flash column chromatography was performed with Aldrich silica gel (60 Å, 230-400 mesh)). High resolution mass spectra (HRMS, ESI) were recorded on an Agilent mass spectrometer using ESI-TOF (Electrospray ionization-time of flight). Thin-layer chromatography (TLC) was performed on Merck silica plates (60F-254) and compounds were visualized by short-wavelength UV light and $KMnO_4$/ninhydrine staining. Preparative HPLC was performed using an Alltima C18 5µ column (22 × 250 mm) and linear gradients of water + 0.1% TFA (eluent A) and MeCN + 0.1% TFA (eluent B) at a flow rate of 10 mL/min., unless stated otherwise. Analytical high-performance liquid chromatography (HPLC) analysis was performed using a Jasco HPLC system equipped with an Alltima C18 5µ column (4.6 × 250 mm) and linear gradients of MeCN/water + 0.1% TFA at a flow rate of 1 mL/min..

Analytical HPLC was performed using an Alltima C18 5µ column (4.6 × 250 mm) and linear gradients of MeCN/H_2O + 0.1% TFA. Size exclusion chromatography (SEC) of conjugates was performed on a Zenix-C sec-300 column (7.8 × 300 mm; 5 µm; Sepax Technologies Inc., Newark, DE, USA) with phosphate buffer (0.05 M, pH 6.6) containing sodium chloride (0.15

M) and sodium azide (0.01 M). LC-MS analysis was performed using a Thermo Finnigan LC system (Thermo Finnigan, San Jose, CA, USA), coupled to a Bruker Q-TOF mass spectrometer (Bremen, Germany) equipped with an electrospray ionization (ESI) source. Intact conjugate mass determination was performed using a Zenix-C column (4.6 × 300 mm; 5 μm; Sepax Technologies Inc., Newark, DE, USA). The mobile phase consisted of a mixture of H_2O, MeCN, TFA, and formic acid (79.9/19.9/0.1/0.1, v/v/v/v, respectively). A 17-min. isocratic run was performed at a flow rate of 350 μL/min.. 10 μL of a sample were injected. In both cases, the LC flow was directed to the MS source from 2 to 10 min. using the switch valve present on the mass spectrometer. The rest of the solvent flow was directed to waste to prevent source contamination. MS analysis was done in positive ionization mode using the following settings: ESI voltage, 4.5 kV; dry gas temperature, 190 °C; dry gas flow rate, 8 L/min.; nebulizer pressure, 1.6 bar; in-source collision-induced dissociation energy, 120 eV; ion energy, 5 eV; collision cell energy, 15 eV. Data was analyzed using Bruker Daltonics Data Analysis software. Protein ion charge assignment and molecular mass determinations were performed using the "Charge Deconvolution" utility of the Data Analysis software. Elemental analyses were performed at UCD School of Chemistry and Chemical Biology Microanalytical Laboratory University College Dublin using a CE 440 elemental analyser.

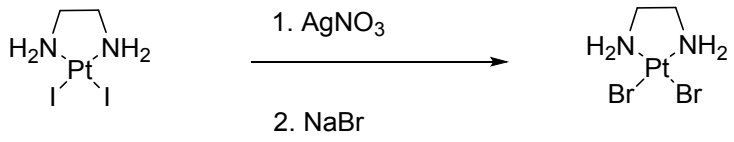
1.2. General information on the optimization of the "complexation" reaction using noreleagnine (1a) as the model compound

The optimization experiments were performed on a 50 μmol scale, using oil bath or a Thermoshaker, and the reaction parameters were varied as specified in the experiment description. Temperature was increased step wise and the reaction mixtures were kept at a certain temperature for time indicated in the tables. Samples were taken and the conversion was determined by HPLC. For these optimization experiments, no isolation of the products was performed, unless stated otherwise.

Following reaction parameters were screened: solvents (sections 3.3. and 3.4.), organic bases (section 3.5.), stoichiometry (section 3.6.), and concentration (section 3.8.).

2. Synthesis and analytical characterization of *Lx*Hal_2 complexes 2b and 2c

2.1. Synthesis and analytical characterization of the platinum complex *Lx*Br_2 (2b)

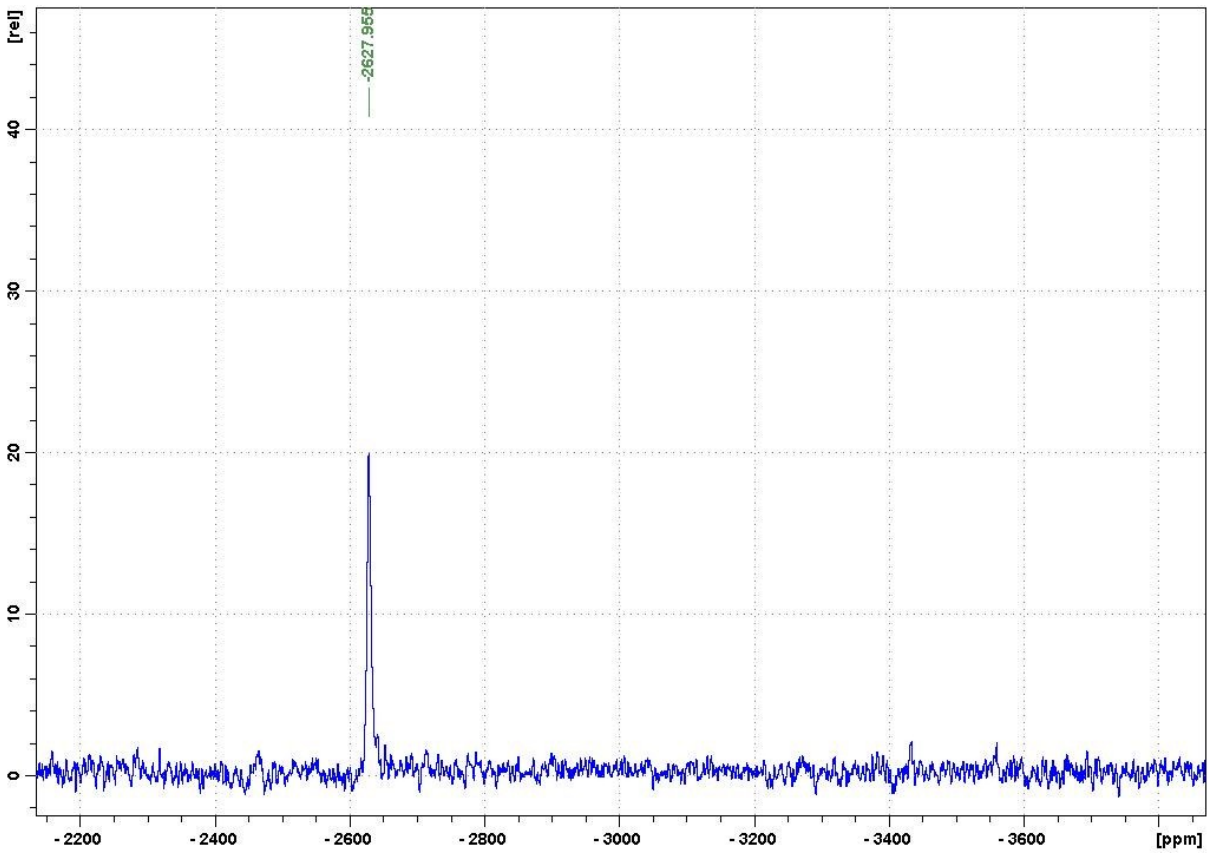


2c **2b**

LxI_2 (**2c**) (509 mg, 1.00 mmol, 1.00 eq.) was added portionwise as a solid to a stirred solution of $AgNO_3$ (339.7 mg, 2.00 mmol, 2.00 eq.) in water (5 mL). The mixture was stirred at room temperature for 24 h in the dark. The AgI precipitate was removed by filtration through Celite, and an excess of NaBr (346 mg, 3.36 mmol, 3.36 eq.) was added to the filtrate (clear light yellow almost colorless filtrate) as a solution in water (1 mL), and the mixture was stirred at room temperature for 20 h. The mixture became yellow upon addition of NaBr and a precipitate started to form after ~1 min. after addition. The yellow precipitate was collected by filtration, thoroughly washed with water, followed by washing with methanol, and dried under reduced pressure. The obtained filter cake (376 mg of a yellow solid) was transferred into a flask and slurry-washed in MeOH (5 mL) for 1 h, collected by filtration, and the filter cake was washed with MeOH, and then dried under reduced pressure for 12 h to obtain a yellow solid (359 mg, 87% yield).

Elemental analysis calc for $C_2H_8Br_2N_2Pt$: C, 5.79; H, 1.94; N, 6.75; found: C, 5.78; H, 1.72; N, 6.54.

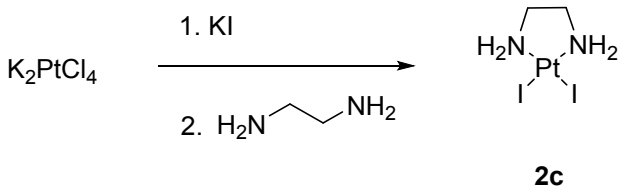
^{195}Pt-NMR (86 MHz, DMF-d_7): δ -2628.



^{195}Pt-NMR spectrum of *Lx*Br_2 (**2b**)

12.5 mg/0.5 mL DMF-d_7, T = 24.1 °C, number of scans: 30000

2.2. Synthesis and analytical characterization of the platinum complex *Lx*I_2 (2c)



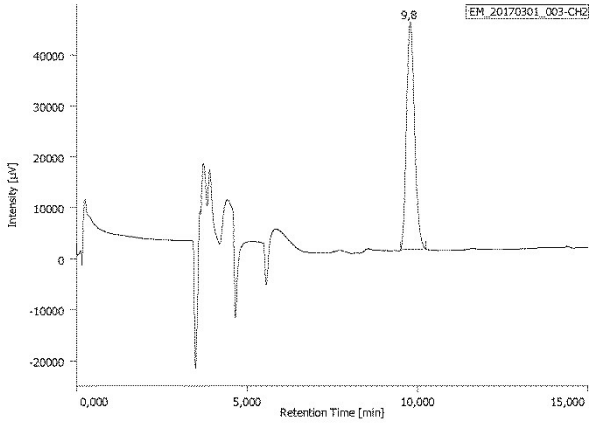
2c

KI (33.2 g, 0.2 mol, 20 eq.) was added to a solution of K_2PtCl_4 (4.15 g, 10 mmol, 1.0 eq.) in water (200 mL). The mixture was stirred at room temperature for 22 h, then the resulting dark mixture was filtered, ethane-1,2-diamine (800 µL, 12 mmol, 1.2 eq.) was added to the filtrate, and the mixture was stirred at room temperature for 23 h. A yellow precipitate started to form immediately upon addition of ethane-1,2-diamine. The precipitate was collected by filtration, thoroughly washed with water, and dried first under suction on the filter for 3-4 h and then under reduced pressure for 12 h to obtain a yellow solid (4.85 g, 95% yield).

HPLC (Grace Alltima C18, 25 × 4.6 mm, 5 µm) indicated that the product was 100% pure (retention time t_R = 9.8 min.; gradient: 5 to 50% MeCN/0.1% TFA in water/0.1% TFA in 18 min. measured at a wavelength of 273 nm).

Report

User Name	NS
Description	EM-0155-1
HPLC System Name	HPLC Systeem 3
Injection Date	1-3-2017 17:37:45
Volume	20,00 [µL]
Sample #	20
Project Name	C18 Alltima_2017
Executed Sequence	Analytical C18 Alltima2
Sample Name	LxI2
Sample Description	QC
Acquisition Sequence	Analytical C18 Alltima2
Control Method	EM_indole_medium polar_223 273 210nm

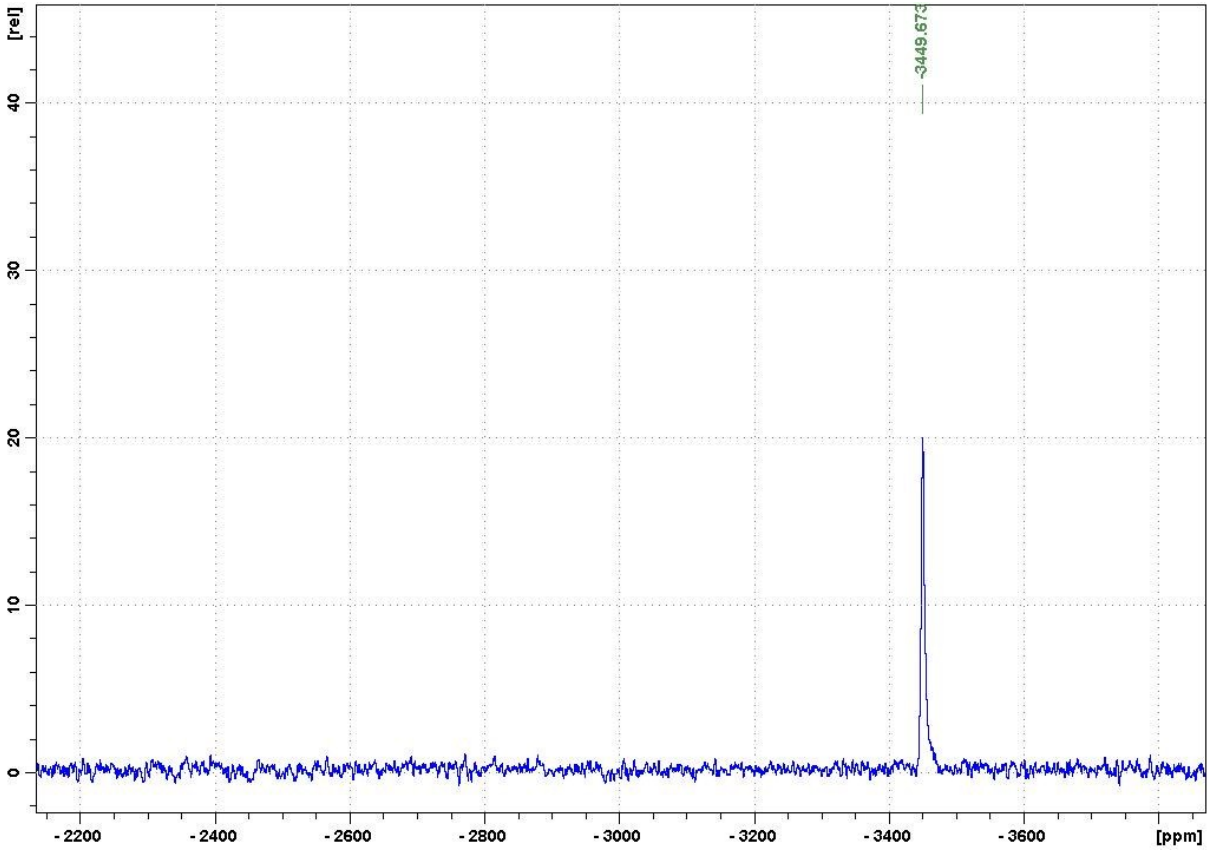


Peak number	Peak Name	Retention time [min]	Area [µV·sec]	Height [µV]	Area%	Symmetry Factor	Resolution
1	Unknown	9,783	730898	44630	100,000	1,182	N/A

The HPLC chromatogram of *Lx*I_2 (**2c**). t_R = 9.8 min.

Elemental analysis calc for $C_2H_8I_2N_2Pt$: C, 4.72; H, 1.58; N, 5.50; found: C, 4.68; H, 1.44; N, 5.30.

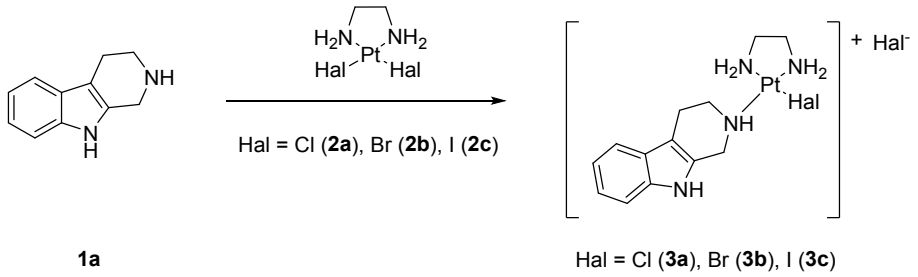
^{195}Pt-NMR (86 MHz, DMF-d_7): δ -3450. Lit (*Inorg. Chem.* **1992**, *31*, p. 5447): -3450.



^{195}Pt-NMR spectrum of *Lx*I_2 (**2c**)

30.0 mg/0.5 mL DMF-d_6, T = 19.8 °C, number of scans: 1500

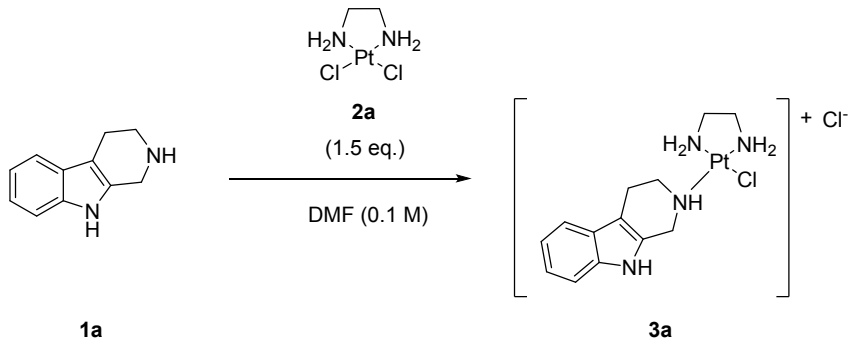
3. Optimization of the "complexation" reaction



The reactions were performed in dry DMF (0.1 M in **1a**) using 1.5 eq. of complexes *Lx*Hal_2 **2a-c**. The reaction mixture was placed into a preheated oil bath at the initial indicated temperature.

3.1. Reaction of noreleagnine (**1a**) with *Lx*Hal_2 complexes **2a-c**

3.1.1. Reaction of noreleagnine (**1a**) with *Lx*Cl_2 complex **2a**



Supplementary Scheme 1: Formation of the chlorido "semi-final" complex **3a**

The reaction progress (conversion followed on HPLC, definied as [a/a% (**3a**) / a/a% (**1a** + **3a**) × 100]% at 273 nm) was followed according to the temperature program (temperature was

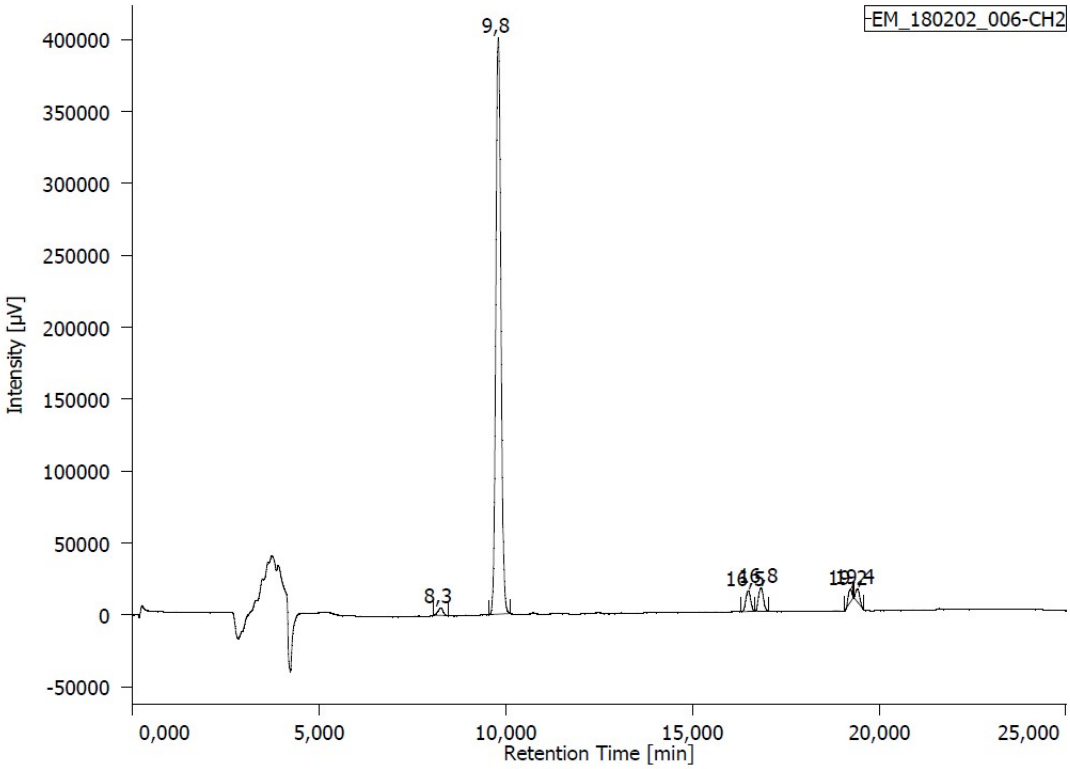
increased step-wise; the oil bath temperature is indicated) which is depicted in Supplementary Table 1.

Supplementary Table 1: Conversion of noreleagnine **1a** (t_R = 8.2 min.) to the chlorido "semi-final" complex **3a** (t_R = 9.8 min.), measured at 273 nm at different temperatures at indicated time points

T, time	40 °C, 20 h	60 °C, 24 h	80 °C, 24 h	100 °C, 2 h
a/a% (1a)	87.4	13.3	**1.2**	2.9
a/a% (3a)	10.4	72.4	**89.2**	84.9
conversion [%] [a/a% (3a) / a/a% (1a + 3a)] × 100	10.6	84.5	**98.7**	96.7
observations	yellow heterogeneous mixture			

The product formation was confirmed by HRMS: $C_{13}H_{20}{}^{35}ClN_4{}^{195}Pt^+$ $[M]^+$: calc 462.1020; found: 462.1041.

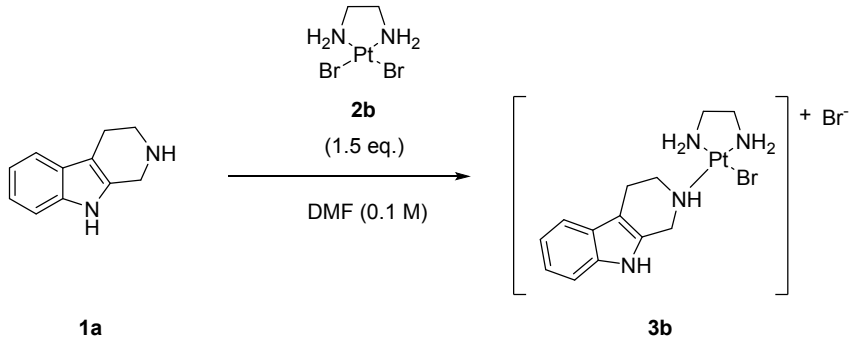
The best conversion was observed at 80 °C (Supplementary Figure 1).



Peak number	Peak Name	Retention time [min]	Area [µV·sec]	Height [µV]	Area%	Symmetry Factor	Resolution
1	Unknown	8,258	50071	5282	1,186	0,907	6,263
2	Unknown	9,800	3767209	400697	89,199	1,058	27,183
3	Unknown	16,483	127680	14245	3,023	1,046	1,382
4	Unknown	16,825	148295	16348	3,511	1,088	10,634
5	Unknown	19,217	63228	8977	1,497	0,850	0,931
6	Unknown	19,408	66892	9013	1,584	1,173	N/A

Supplementary Figure 1: The HPLC chromatogram of the reaction of noreleagnine **1a** to the complex **3a** at 80 ^{o}C/24 h (after being reacted for 20 h at 40 ^{o}C, followed by 24 h at 60 ^{o}C), monitored at 273 nm

3.1.2. Reaction of noreleagnine (**1a**) with *Lx*Br_2 complex **2b**



Supplementary Scheme 2: Formation of the bromido "semi-final" complex **3b**

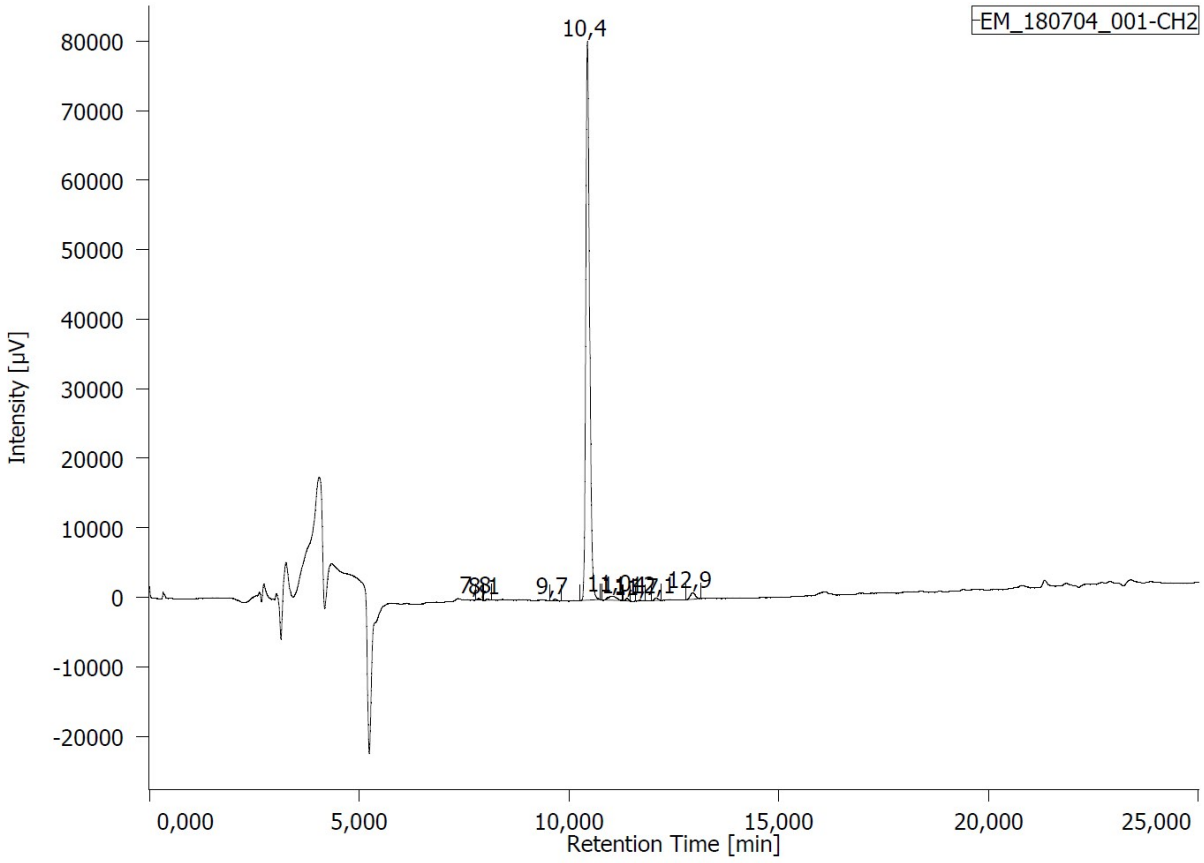
The reaction progress (conversion followed on HPLC, definied as [a/a%(**3b**) / a/a% (**1a** + **3b**)] at 273 nm) was followed according to the temperature program (temperature was increased step-wise; the oil bath temperature is indicated) depicted in Supplementary Table 2.

Supplementary Table 2: Conversion of noreleagnine **1a** (t_R = 8.1 min.) to the bromido "semi-final" complex **3b** (t_R = 10.4 min.), measured at 273 nm at different temperatures at indicated time points

T, time	40 °C, 20 h	60 °C, 24 h	80 °C, 24 h	100 °C, 24 h
a/a% (1a)	1.2	**0.2**	1.4	2.4
a/a% (3b)	95.4	**95.7**	90.3	93.1
conversion [%] **[a/a% (3b) / a/a% (1a + 3b)] ×** **100**	98.8	**99.8**	98.5	98.1

observations	yellow homogeneous mixture (became homogeneous after stirring at 40 °C for several hours)

The best conversion was observed at 60 °C (Supplementary Figure 2).

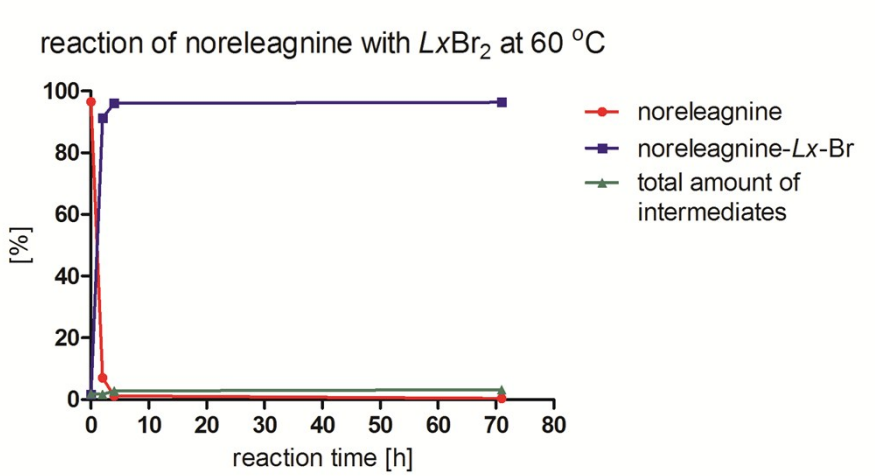


Peak number	Peak Name	Retention time [min]	Area [µV·sec]	Height [µV]	Area%	Symmetry Factor	Resolution
1	Unknown	7,842	968	185	0,180	1,299	1,495
2	Unknown	8,058	920	177	0,171	1,214	11,420
3	Unknown	9,667	1239	244	0,230	1,141	5,090
4	Unknown	10,442	515471	80350	95,682	1,233	2,131
5	Unknown	11,033	8020	583	1,489	1,078	1,296
6	Unknown	11,375	1689	339	0,314	1,110	1,798
7	Unknown	11,675	611	92	0,113	1,325	2,296
8	Unknown	12,075	1953	353	0,362	1,178	4,630
9	Unknown	12,942	7865	912	1,460	1,150	N/A

Supplementary Figure 2: The HPLC chromatogram of the reaction of noreleagnine **1a** to the complex **3b** at 60 °C/24 h (after being reacted for 20 h at 40 °C), monitored at 273 nm.

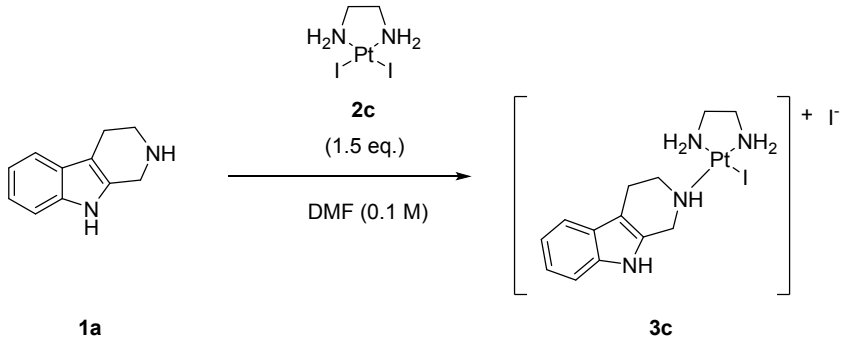
The product formation was confirmed by HRMS: $C_{13}H_{20}{}^{79}BrN_4{}^{195}Pt^+$ $[M]^+$: calc 506.0514; found: 506.0494.

Then, the reaction was repeated at 60 °C and the reaction monitoring of converting the starting material **1a** into the product **3b** was performed (Supplementary Figure 3). After 2 h, the initially heterogeneous reaction mixture became homogeneous.



Supplementary Figure 3: Reaction monitoring for reaction time = 0 h to 71 h.

3.1.3. Reaction of noreleagnine (**1a**) with $Lx I_2$ complex **2c**



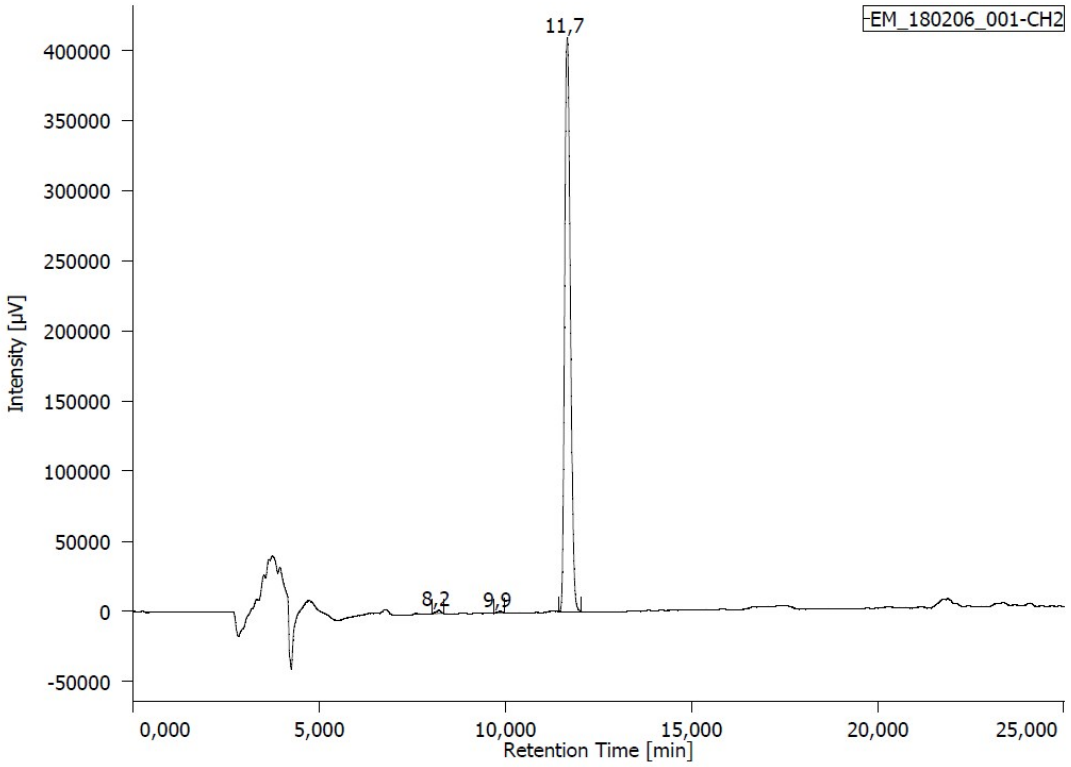
Supplementary Scheme 3: Formation of the iodido "semi-final" complex **3c**

The reaction progress (conversion followed on HPLC, definied as [a/a%(**3c**) / a/a% (**1a** + **3c**)] × 100% at 273 nm) was followed according to the temperature program (temperature was increased step-wise; the oil bath temperature is indicated) depicted in Supplementary Table 3.

Supplementary Table 3: Conversion of noreleagnine **1a** (t_R = 8.2 min.) to the iodido "semi-final" complex **3c** (t_R = 11.7 min.), measured at different temperatures at 273 nm at indicated time points

T, time	40 °C, 20 h	60 °C, 24 h	80 °C, 24 h	90 °C, 3 h
a/a% (1a)	**0.4**	1.2	2.8	4.2
a/a% (3c)	**99.3**	98.6	96.9	94.5
conversion [%] **[a/a% (3c) / a/a% (1a + 3c)] × 100**	**99.6**	98.8	97.2	95.7
observations	yellow homogeneous mixture (it became homogeneous already at start of the reaction)			

The best conversion was observed at 40 °C (Supplementary Figure 4).

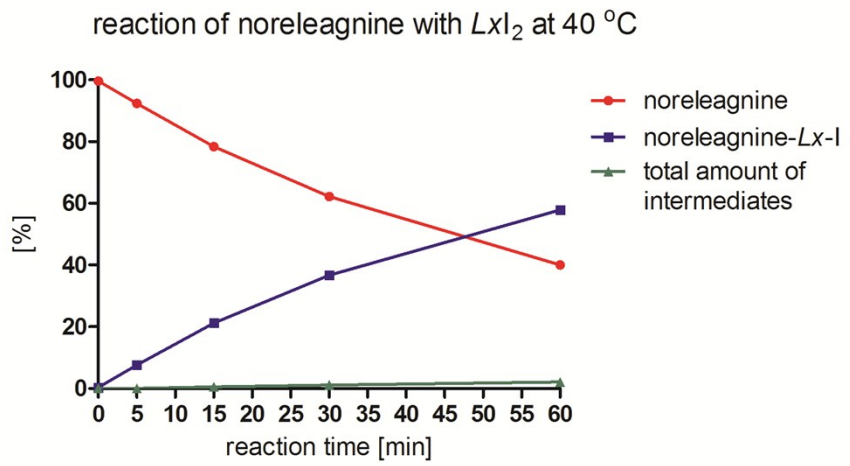


Peak number	Peak Name	Retention time [min]	Area [µV·sec]	Height [µV]	Area%	Symmetry Factor	Resolution
1	Unknown	8,208	19282	2182	0,448	0,894	6,862
2	Unknown	9,858	9012	1052	0,209	0,872	6,924
3	Unknown	11,658	4279781	409346	99,343	1,201	N/A

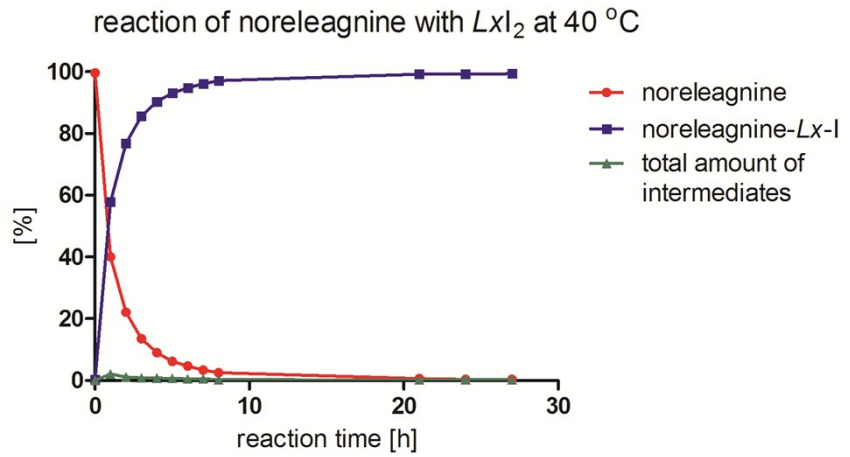
Supplementary Figure 4: The HPLC chromatogram of the reaction of noreleagnine **1a** to the complex **3c** at 40 °C/20 h, monitored at 273 nm

The product formation was confirmed by HRMS: $C_{13}H_{20}IN_4{}^{195}Pt^+$ $[M]^+$: calc 554.0376; found: 554.0371.

Then, the reaction was repeated at 40 °C and the reaction monitoring of converting the starting material **1a** into the product **3c** was performed (Supplementary Figure 5 and Supplementary Figure 6).

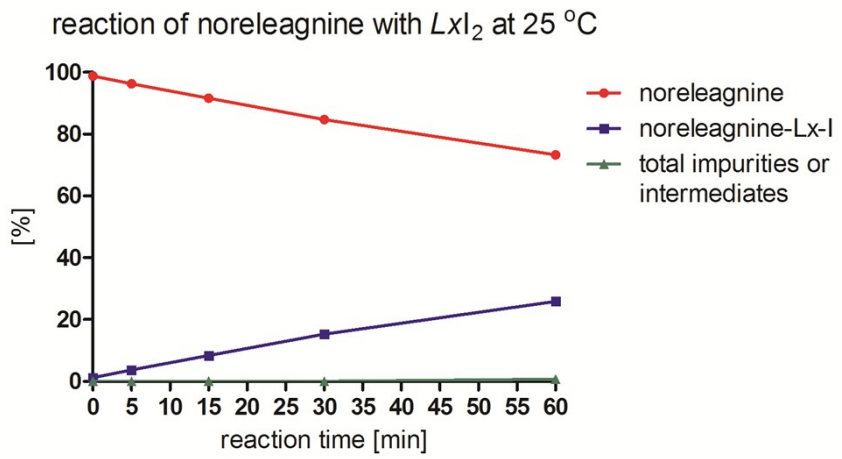


Supplementary Figure 5: Reaction monitoring for reaction time = 0 min. to 60 min.

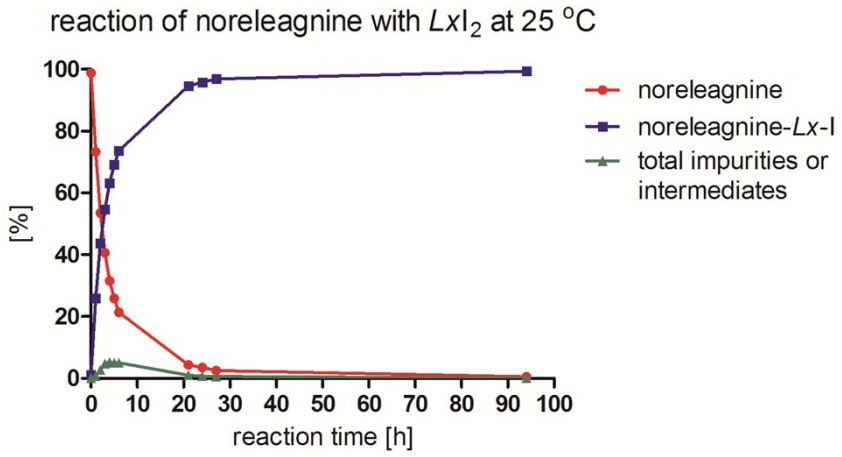


Supplementary Figure 6: Reaction monitoring for reaction time = 0 h to 27 h.

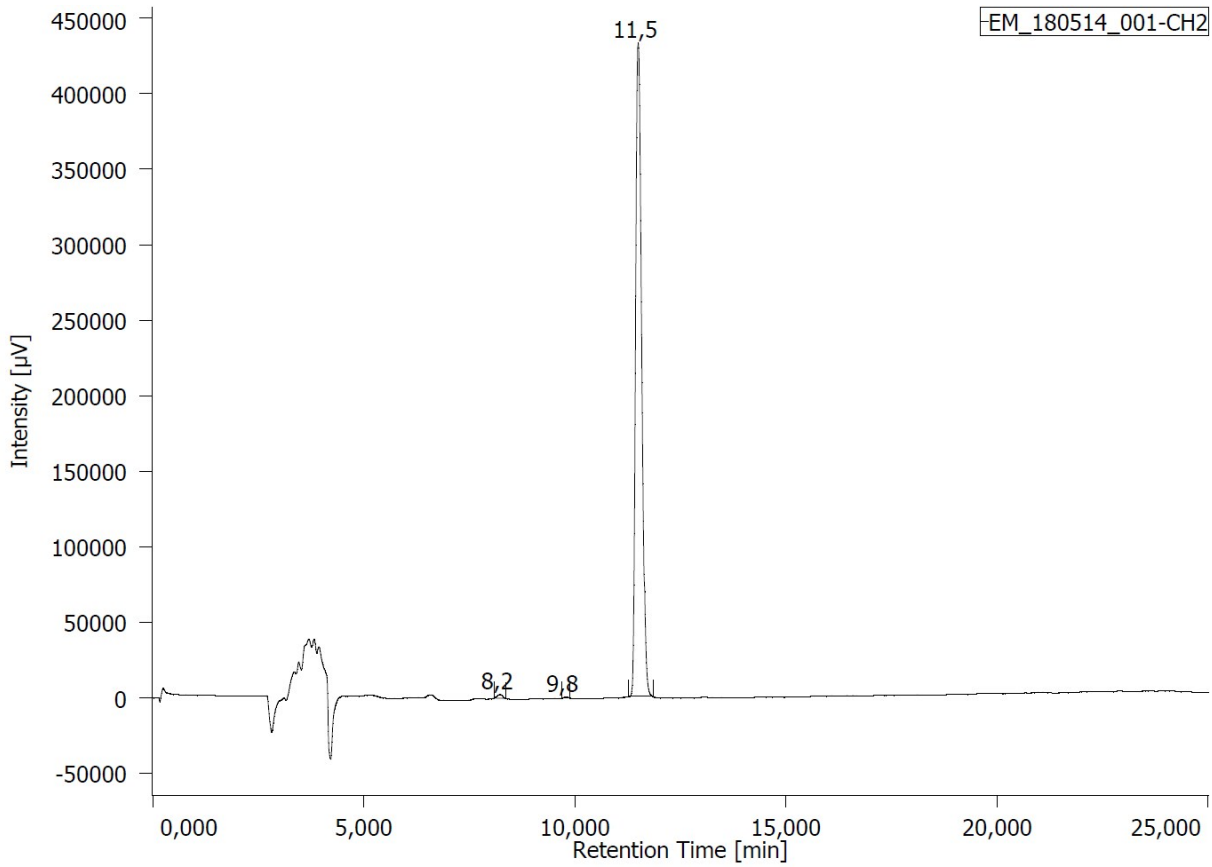
Additionally, regarding the almost completeness of the reaction at 40 °C after 20 h, the reaction progress was monitored at 25 °C and samples were measured at time points between 5 min. up to 94 h (Supplementary Figure 7 and Supplementary Figure 8).



Supplementary Figure 7: Reaction monitoring for reaction time = 0 min. to 60 min.



Supplementary Figure 8: Reaction monitoring for reaction time = 0 h to 94 h.



Peak number	Peak Name	Retention time [min]	Area [µV·sec]	Height [µV]	Area%	Symmetry Factor	Resolution
1	Unknown	8,233	21374	2485	0,493	0,907	6,983
2	Unknown	9,783	6278	864	0,145	1,042	7,283
3	Unknown	11,500	4304250	432463	99,362	1,265	N/A

Supplementary Figure 9: The HPLC chromatogram of the reaction of noreleagnine **1a** to the complex **3c** at 25 °C/94 h, monitored at 273 nm.

After 94 h at 25 °C, the mixture was diluted with water (10 mL) and filtered through filter paper to remove the precipitated *Lx*I_2 (**2c**) complex. The HPLC analysis detected 0.6 a/a% of starting material **1a** and 99.4% of product **3c** with no *Lx*I_2 (**2c**) present. Then, the mixture was purified by reverse-phase chromatography using a short colomn (500 mg) of RP-C18 (Merck LiChroprep (15-25 µm)). The colomn was pre-washed with MeOH (3 × 1 mL). The mixture was applied on the column, washed with water/MeOH 9:1 (10 mL), then eluted with

water/MeOH 8:2 (5 mL), and finally with water/MeOH 2:8 (4 mL). In total, ~29 mg (85% yield) of product were obtained. The purest fraction (which was eluted with water/MeOH 8:2) contained 18 mg of a yellow solid (53% yield). NaI (3.6 mg), dissolved in ^{t}BuOH/water 1:1, was added to stabilize the product towards hydrolysis, and the mixture was lyophilized. HPLC revealed the product purity of 98.5%.

3.2. Addition of potassium iodide into the reaction mixture of noreleagnine (**1a**) with *Lx*I_2 complex (**2c**)

The reactions were performed in parallel in a Thermoshaker (550 rpm). In HPLC vials, noreleagnine (**1a**) (8.6 mg, 50 µmol, 1.0 eq.), KI (amounts are indicated in Supplementary Table 4), and *Lx*I_2 (**2c**) (25.4 mg, 50 µmol, 1.0 eq.) were placed at 25 °C in dry DMF (500 µL) under light exclusion and under argon atmosphere. The temperature of the Thermoshaker was raised step-wise according to Supplementary Table 4; the speed of the Thermoshaker was 550 rpm.

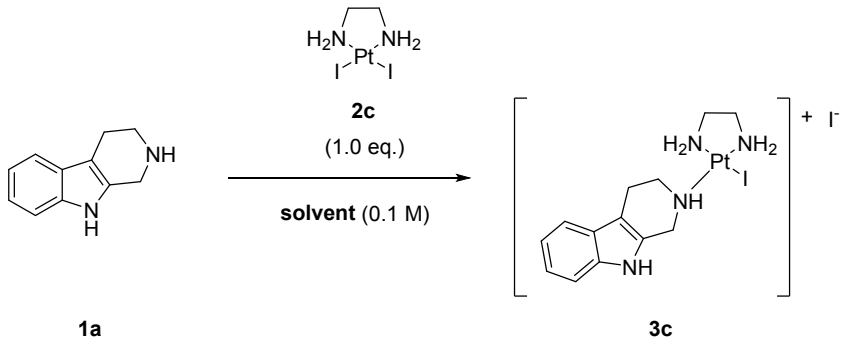
The reaction progress (conversion followed on HPLC, definied as [a/a% (**3c**) / a/a% (**1a** + **3c**)] × 100% at 273 nm) is depicted in Supplementary Table 4. The retention time of the starting material noreleagnine (**1a**) was t_R = 8.1 min. and the retention time of the product complex **3c** was t_R = 11.5 min..

Supplementary Table 4: Addition of potassium iodide into the reaction mixture of noreleagnine (**1a**) with *Lx*I_2 complex (**2c**)

amount KI (eq.)	conversion at 25 °C, 69 h (%)	conversion at 40 °C, 24 h (%)	conversion at 60 °C, 24 h (%)	conversion at 80 °C, 6 h (%)
0.0	94.1	95.4	92.4	87.1
0.2	93.8	95.0	92.1	86.1
0.4	94.0	95.2	91.9	86.1
0.6	94.6	95.9	92.7	86.7
0.8	91.8	92.7	89.2	83.5
1.0	92.7	94.2	90.6	84.5

2.0	94.0	95.1	91.1	84.8
5.0	92.7	93.1	88.6	81.7

3.3. Screening of solvents for the reaction of noreleagnine (**1a**) with *Lx*I_2 complex (**2c**)



Supplementary Scheme 4: The solvent screening.

The reactions were performed in parallel in a Thermoshaker. In HPLC vials, noreleagnine (**1a**) (8.6 mg, 50 μmol, 1.0 eq.) and *Lx*I_2 (**2c**) (25.4 mg, 50 μmol, 1.0 eq.) were placed at 25 °C in a dry solvent under light exclusion and under argon atmosphere, according to Supplementary Table 5. The temperature of the Thermoshaker was kept at 25 °C; the speed of the Thermoshaker was 550 rpm.

The reaction progress (conversion followed on HPLC, definied as [a/a% (**3c**) / a/a% (**1a** + **3c**)] × 100% at 273 nm) is depicted in Supplementary Table 5. The retention time of the starting material noreleagnine (**1a**) was t_R = 8.1 min. and the retention time of the product complex **3c** was t_R = 11.5 min..

The product **3c** was isolated by preparative HPLC from three best "complexation" mixtures.

Isolation of the reaction mixture in DMF:

To the reaction mixture, 20% MeOH/water solution (2.5 mL) was added, resulting in a homogeneous mixture. After filtration through the syringe filter, the purification was performed by preparative reverse-phase HPLC (gradient: 20-100% 95% MeOH/5% water/0.1% TFA in 95% water/5% MeOH/0.1% TFA in 40 min..). After lyophilization of the product containing fractions, the product **3c** was obtained as a colorless solid (24 mg, 35.2 µmol, 70% yield).

HPLC (Grace Alltima C18 5 µm column, 25 × 4.6 mm) indicated that the product was 96.5% pure (retention time t_R = 11.4 min.; gradient: 20-100% MeCN/0.1% TFA in water/0.1% TFA in 20 min., measured at a wavelength of 273 nm).

Isolation of the reaction mixture in DMA:

To the reaction mixture, 20% MeOH/water solution (2.5 mL) was added, resulting in a homogeneous mixture. After filtration through the syringe filter, the purification was performed by preparative reverse-phase HPLC (gradient: 20-100% 95% MeOH/5% water/0.1% TFA in 95% water/5% MeOH/0.1% TFA in 40 min.). After lyophilization of the product containing fractions, the product **3c** was obtained as a colorless solid (24 mg, 35.2 µmol, 70% yield).

HPLC (Grace Alltima C18 5 µm column, 25 × 4.6 mm) indicated that the product was 97.1% pure (retention time t_R = 11.4 min.; gradient: 20-100% MeCN/0.1% TFA in water/0.1% TFA in 20 min., measured at a wavelength of 273 nm).

Isolation of the reaction mixture in DMSO:

To the reaction mixture, 45% MeOH/water solution (2.5 mL) was added, resulting in a homogeneous mixture. After filtration through the syringe filter, the purification was performed by preparative reverse-phase HPLC (gradient: 45-80% 95% MeOH/5% water/0.1% TFA in 95%water/5% MeOH/0.1% TFA in 40 min.). After lyophilization of the product containing fractions, the product **3c** was obtained as a colorless solid (16 mg, 35.2 µmol, 47% yield).

HPLC (Grace Alltima C18 5 µm column, 25 × 4.6 mm) indicated that the product was 76.7% pure (retention time t_R = 11.5 min.; gradient: 20-100% MeCN/0.1% TFA in water/0.1% TFA in 20 min., measured at a wavelength of 273 nm).

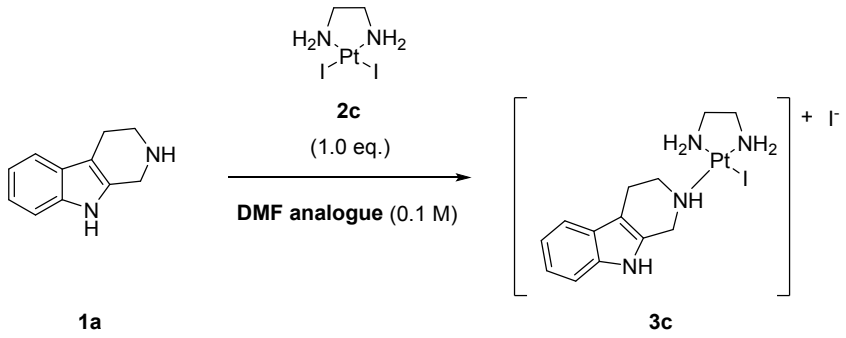
Supplementary Table 5: Screening of solvents for the reaction of noreleagnine (**1a**) with *Lx*I_2 complex (**2c**)

solvent	**reaction time (h)**					
	0 (start)	**16**	**24**	**48**	**66**	**158**
DMF	0.7	73.9	82.1	91.1	93.9	95.4*
DMF + 1 vol% water	0.7	72.8	80.7	89.9	92.8	94.5
DMF + 10 vol% water	0.7	68.1	76.9	87.9	91.8	94.3
DMA	0.0	66.5	77.3	88.7	91.7	95.2*
MeOH	0.0	1.0	1.3	2.6	-	-
EtOH	0.0	0.0	0.4	0.9	-	-
MeCN	0.0	16.1	23.8	23.6	-	-

DMSO	0.0	73.9	73.5	75.5	81.2	81.3
acetone	0.0	12.5	12.0	16.1	-	-
ethyl acetate	0.0	1.1	0.6	0.5	-	-
NMP	4.5	62.4	73.5	84.0	86.1	88.2*
DMI	1.2	64.3	73.8	82.7	84.1	85.5*
DMPU	1.0	55.3	65.8	79.6	82.5	85.7*

*The reaction time was 136 h instead of 158 h

3.4. Screening of DMF analogues as solvents for the reaction of noreleagnine (**1a**) with *Lx*I_2 complex (**2c**)



Supplementary Scheme 5: The screening of DMF analogues as solvents.

The reactions were performed in parallel in a Thermoshaker. In HPLC vials, noreleagnine (**1a**) (8.6 mg, 50 µmol, 1.0 eq.) and *Lx*I_2 (**2c**) (25.4 mg, 50 µmol, 1.0 eq.) were placed at 25 °C in a DMF analogue as a solvent under light exclusion and under argon atmosphere, according to Supplementary Table 6. The temperature of the Thermoshaker was kept at 25 °C; the speed of the Thermoshaker was 550 rpm.

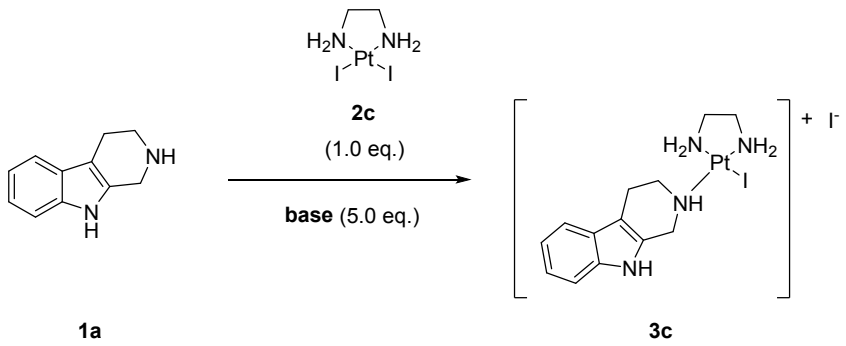
The reaction progress (conversion followed on HPLC, definied as [a/a% (**3c**) / a/a% (**1a** + **3c**)] × 100% at 273 nm) is depicted in Supplementary Table 6. The retention time of the starting material noreleagnine (**1a**) was t_R = 8.1 min. and the retention time of the product complex **3c** was t_R = 11.5 min..

Supplementary Table 6: Screening of DMF analogues as solvents for the reaction of noreleagnine (**1a**) with $Lx I_2$ complex (**2c**)

DMF analogue	**reaction time (h)**				
	0 (start)	**16**	**24**	**45**	**136**
DMF*	0.0	78.0	83.6	87.7	95.3
N-formyl pyrrolidine	0.0	71.0	78.5	85.3	94.6
N-formyl piperidine	0.0	76.6	82.5	87.7	93.6
N-formyl morpholine	0.0	82.2	87.0	90.6	95.2
diethyl formamide	0.0	47.6	55.3	66.7	_
diisopropyl formamide	0.0	24.9	29.8	37.4	_
methyl formamide	0.0	71.5	76.5	79.3	91.3
formamide	0.0	13.4	14.2	18.9	_
tetramethyl urea	0.0	61.3	70.1	80.3	89.8

*DMF was used as a standard for comparison

3.5. Screening of organic bases for the reaction of noreleagnine (**1a**) with *Lx*I_2 complex (**2c**)



Supplementary Scheme 6: The base screening.

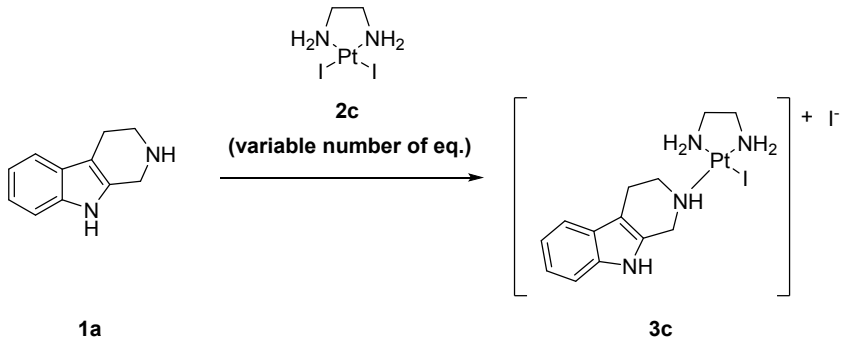
The reactions were performed in parallel in a Thermoshaker. In HPLC vials, noreleagnine (**1a**) (8.6 mg, 50 μmol, 1.0 eq.) and *Lx*I_2 (**2c**) (25.4 mg, 50 μmol, 1.0 eq.) were placed at 25 °C in dry DMF (500 μL) under light exclusion and under argon atmosphere. Then, the base (5.0 eq.) was added, according to Supplementary Table 7. *Note*: DABCO as a solid base was added before the addition of DMF. The temperature of the Thermoshaker was kept at 25 °C; the speed of the Thermoshaker was 550 rpm.

The reaction progress (conversion followed on HPLC, definied as [a/a% (**3c**) / a/a% (**1a** + **3c**)] × 100% at 273 nm) is depicted in Supplementary Table 7. The retention time of the starting material noreleagnine (**1a**) was t_R = 8.1 min. and the retention time of the product complex **3c** was t_R = 11.5 min..

Supplementary Table 7: Screening of organic bases for the reaction of noreleagnine (**1a**) with *Lx*I_2 complex (**2c**)

base	**reaction time (h)**				
	0 (start)	**16**	**24**	**48**	**96**
no base (control)	0.0	76.8	83.9	91.2	94.6
TEA	0.0	49.1	62.6	77.0	85.6
DIPEA	0.0	29.4	44.4	64.6	78.0
N-Me piperidine	0.0	61.0	70.9	81.6	87.0
N-Me morpholine	0.0	75.6	82.3	89.8	93.8
DBU	0.0	0.3	0.0	0.0	0.0
DABCO	0.0	47.2	66.0	78.9	78.1
2,6-lutidine	0.0	75.8	82.0	89.3	92.7
2,4,6-collidine	0.0	76.1	82.0	89.5	93.7

3.6. The stoichiometry screening for the reaction of noreleagnine (**1a**) with $Lx I_2$ complex (**2c**)



Supplementary Scheme 7: The stoichiometry screening.

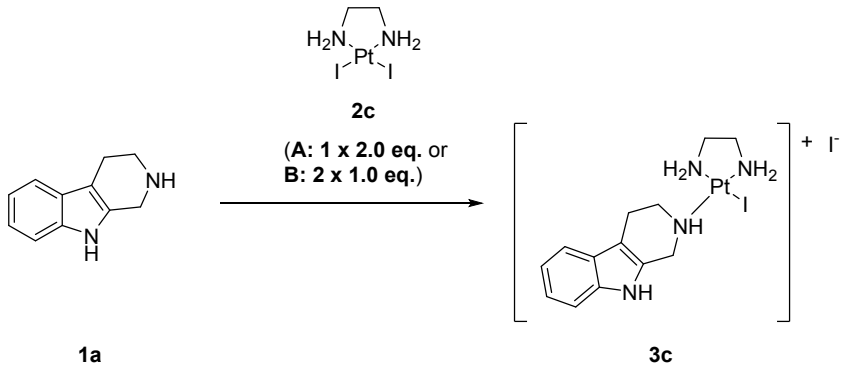
The reactions were performed in parallel in a Thermoshaker. In HPLC vials, noreleagnine (**1a**) (8.6 mg, 50 µmol, 1.0 eq.) and $Lx I_2$ (**2c**) (number of eq. according to Supplementary Table 8) were placed at 25 °C in dry DMF (500 µL) under light exclusion and under argon atmosphere. The temperature of the Thermoshaker was kept at 25 °C; the speed of the Thermoshaker was 550 rpm.

The reaction progress (conversion followed on HPLC, definied as [a/a% (**3c**) / a/a% (**1a** + **3c**)] × 100% at 273 nm) is depicted in Supplementary Table 8. The retention time of the starting material noreleagnine (**1a**) was t_R = 8.1 min. and the retention time of the product complex **3c** was t_R = 11.5 min..

Supplementary Table 8: The stoichiometry screening for the reaction of noreleagnine (**1a**) with *Lx*I_2 complex (**2c**)

***Lx*I_2 (2c) [eq.]**	**reaction time (h)**							
	0 (start)	**2**	**19**	**24**	**48**	**72**	**96**	**144**
1.0	0.3	27.5	80.4	84.1	92.4	95.1	94.4	97.2
1.1	0.3	29.9	82.8	86.3	93.9	96.5	96.3	98.4
1.2	0.3	32.6	86.3	89.6	96.1	98.1	98.3	99.3
1.5	0.4	40.5	92.8	95.1	98.9	99.7	99.6	99.7
2.0	0.6	51.3	97.3	98.4	99.7	99.8	99.8	99.9
3.0	1.0	66.9	99.4	99.7	99.9	99.9	99.9	99.9
4.0	1.1	76.7	99.7	99.8	99.9	99.9	99.9	99.9
5.0	1.3	82.5	99.9	99.8	100	100	100	99.9

3.7. Influence of one-batch vs two-batch wise addition of *Lx*I_2 (**2c**) on the conversion in the reaction of noreleagnine (**1a**) with *Lx*I_2 complex (**2c**)



Supplementary Scheme 8: The addition mode: one-batch vs two-batch wise addition of *Lx*I_2 (**2c**).

Two reactions were performed in parallel in a Thermoshaker. In HPLC vials, noreleagnine (**1a**) (8.6 mg, 50 µmol, 1.0 eq.) and *Lx*I_2 (**2c**) (reaction **A**: 2.0 eq., 50.9 mg; reaction **B**: 1.0 eq., 25.4 mg) were placed at 25 °C in dry DMF (500 µL) under light exclusion and under argon atmosphere. The temperature of the Thermoshaker was kept at 25 °C; the speed of the Thermoshaker was 550 rpm. After 24 h, another 1.0 eq. (25.4 mg) of *Lx*I_2 (**2c**) was added to the reaction **B** and both reactions were continued for another 24 h.

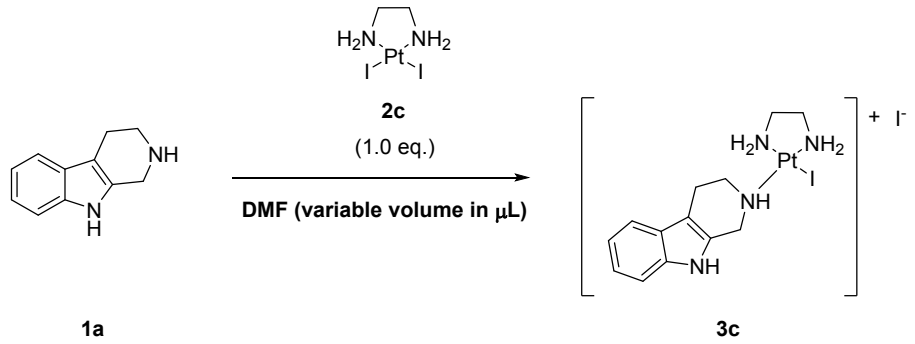
The reaction progress (conversion followed on HPLC, definied as [a/a% (**3c**) / a/a% (**1a** + **3c**)] × 100% at 273 nm) is depicted in Supplementary Table 9. The retention time of the starting material noreleagnine (**1a**) was t_R = 8.1 min. and the retention time of the product complex **3c** was t_R = 11.5 min..

Supplementary Table 9: Influence of one-batch vs two-batch wise addition of *Lx*I_2 (**2c**) on the conversion in the reaction of noreleagnine (**1a**) with *Lx*I_2 complex (**2c**)

reaction mixture	**reaction time (h)**						
	0 (start)	**4**	**19**	**24***	**28**	**42**	**48**
A	0.4	71.6	97.7	98.3	99.3	99.7	99.7
B	0.0	44.0	80.5	84.0	93.8	99.0	99.3

*at this point, 1.0 eq. of *Lx*I_2 (**2c**) were added to the reaction mixture **B**

3.8. The concentration screening for the reaction of noreleagnine (**1a**) with *Lx*I_2 complex (**2c**)



Supplementary Scheme 9: The concentration screening.

The reactions were performed in parallel in a Thermoshaker. In HPLC vials, noreleagnine (**1a**) (8.6 mg, 50 µmol, 1.0 eq.) and *Lx*I_2 (**2c**) (25.4 mg, 50 µmol, 1.0 eq.) were placed at 25 °C in dry DMF (volume according to Supplementary Table 9) under light exclusion and under argon atmosphere. The temperature of the Thermoshaker was kept at 25 °C; the speed of the Thermoshaker was 550 rpm.

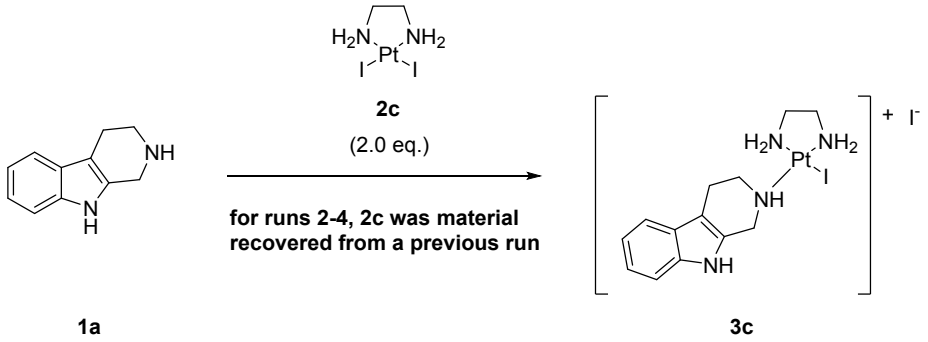
The reaction progress (conversion followed on HPLC, definied as [a/a% (**3c**) / a/a% (**1a** + **3c**)] × 100% at 273 nm) is depicted in Supplementary Table 10. The retention time of the starting material noreleagnine (**1a**) was t_R = 8.1 min. and the retention time of the product complex **3c** was t_R = 11.5 min..

Supplementary Table 10: The concentration screening for the reaction of noreleagnine (**1a**) with *Lx*I_2 complex (**2c**)

V (DMF) / concentration (**1a**)	**reaction time (h)**					
	0 (start)	**24**	**48**	**72**	**96**	**144**
50 µL / 1 M	0.5	95.1	97.1	95.1	96.7	97.3
100 µL / 0.5 M	0.3	95.8	97.1	96.3	97.2	97.8
200 µL / 0.25 M	0.3	92.8	95.3	95.1	96.3	97.1
300 µL / 0.167 M	0.0	89.5	93.3	93.6	95.0	96.2
400 µL / 0.125 M	0.0	87.6	93.1	94.0	95.6	97.0
500 µL / 0.1 M	0.0	84.6	91.2	92.7	94.5	96.2
600 µL / 0.083 M	0.0	81.3	89.0	90.8	93.1	94.9
700 µL / 0.071 M	0.0	79.0	87.8	90.2	92.4	94.5
800 µL / 0.063 M	0.0	76.8	86.4	89.4	91.9	94.3
900 µL / 0.056 M	0.0	74.0	84.7	88.0	90.6	93.3

1000 µL / 0.05 M	0.0	71.8	83.4	87.0	89.6	92.8

3.9. The reaction of noreleagnine (**1a**) with recovered *Lx*I_2 complex (**2c**)



Supplementary Scheme 10: The runs with recovered *Lx*I_2 (**2c**).

The reactions were performed in a preheated oil bath. In a suitable flask (depending on the scale of the run), noreleagnine (**1a**) (1.0 eq.) and *Lx*I_2 (**2c**) (2.0 eq.) were placed at 25 °C in dry DMF (volume according to the scale of the run; all reactions were 0.1 M in the substrate **1a**) under light exclusion and under argon atmosphere.

The reaction progress (conversion followed on HPLC, definied as [a/a% (**3c**) / a/a% (**1a** + **3c**)] × 100% at 273 nm) was determined after 24 h reaction time (*vide infra*). The retention time of the starting material noreleagnine (**1a**) was t_R = 8.1 min. and the retention time of the product complex **3c** was t_R = 11.5 min..

The excessive *Lx*I_2 (**2c**) was recovered by simple precipitation and after washing and drying it was used in the next run. The details on individual runs are described below.

1st run:

In a round-bottom flask, noreleagnine (**1a**) (86.1 mg, 0.5 mmol, 1.0 eq.) and *Lx*I_2 (**2c**) (509 mg, 1.0 mmol, 2.0 eq.) were placed at 25 °C in dry DMF (5 mL) under light exclusion and under argon atmosphere. After 24 h reaction time, the conversion was 99.3%, as measured by HPLC.

After 24 h, the reaction was stopped by adding water (5 mL). After stirring for 0.5 h, the formed precipitate was filtered. The filtrate contained only starting material **1a** (1.0%) and product **3c** (99.0%).

The filter cake was washed thoroughly with water and dried under reduced pressure. Yellow solid (203 mg, ~80% recovered) was obtained and used in *2nd run*. HPLC indicated that the recovered material contained a small amount of product **3c**. No other impurities could be detected.

2nd run:

In a screw-cap bottle, noreleagnine (**1a**) (34.2 mg, 198 μmol, 1.0 eq.) and *Lx*I_2 (**2c**) (202 mg, 397 μmol, 2.0 eq.; recovered after *1st run*) were placed at 25 °C in dry DMF (2 mL) under light exclusion and under argon atmosphere. After 24 h reaction time, the conversion was 98.8%, as measured by HPLC.

After 24 h, the reaction was stopped by adding water (2 mL). After stirring for 0.5 h, the formed precipitate was filtered. The filtrate contained only starting material **1a** (1.2%) and product **3c** (98.8%).

The filter cake was washed thoroughly with water and dried under reduced pressure. Yellow solid (84 mg, ~83% recovered) was obtained and used in *3rd run*. HPLC indicated that the recovered material contained a small amount of product **3c**. No other impurities could be detected.

3rd run:

In an HPLC vial, noreleagnine (**1a**) (14.0 mg, 81.5 µmol, 1.0 eq.) and *Lx*I_2 (**2c**) (83 mg, 163 µmol, 2.0 eq.; recovered after *2nd run*) were placed at 25 °C in dry DMF (800 µL) under light exclusion and under argon atmosphere. After 24 h reaction time, the conversion was 99.3%, as measured by HPLC.

After 24 h, the reaction was stopped by adding water (800 µL). After stirring for 0.5 h, the formed precipitate was filtered. The filtrate contained only starting material **1a** (0.7%) and product **3c** (99.3%).

The filter cake was washed thoroughly with water and dried under reduced pressure. Yellow solid (31 mg, ~75% recovered) was obtained and used in *4th run*. HPLC indicated that the recovered material contained a small amount of product **3c**. No other impurities could be detected.

4th run:

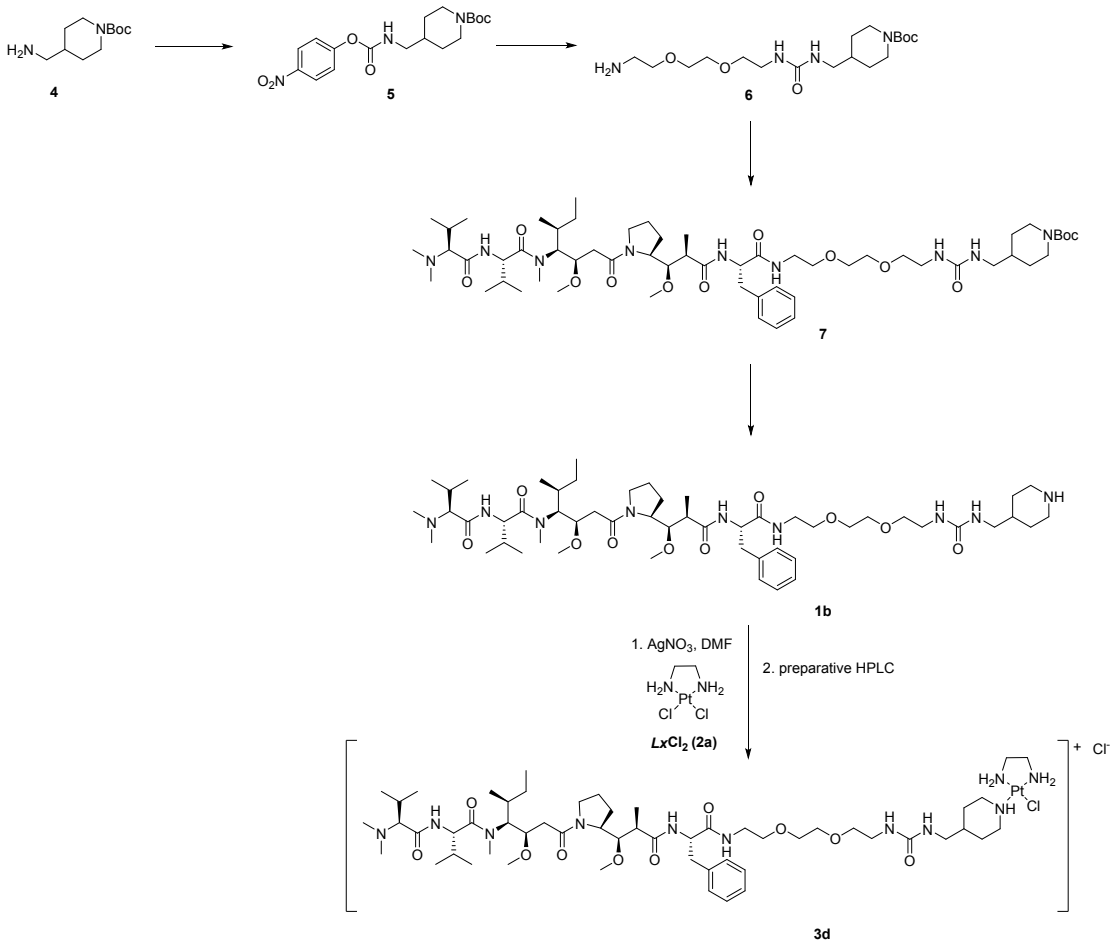
In an HPLC vial, noreleagnine (**1a**) (5.1 mg, 29.5 µmol, 1.0 eq.) and *Lx*I_2 (**2c**) (30 mg, 59 µmol, 2.0 eq.; recovered after *3rd run*) were placed at 25 °C in dry DMF (290 µL) under light exclusion and under argon atmosphere. After 24 h reaction time, the conversion was 99.3%, as measured by HPLC.

After 24 h, the reaction was stopped by adding water (290 µL). After stirring for 0.5 h, the formed precipitate was filtered. The filtrate contained only starting material **1a** (0.7%) and product **3c** (99.3%).

The filter cake was washed thoroughly with water and dried under reduced pressure. Yellow solid (8 mg, ~53% recovered) was obtained. HPLC indicated that the recovered material contained a small amount of product **3c**. No other impurities could be detected.

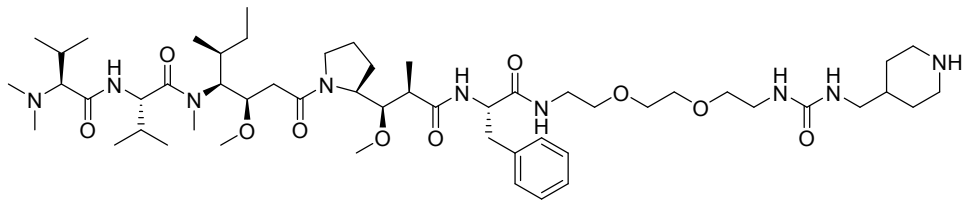
No accumulation of impurities was observed from runs 1 to 4.

4. Initial laboratory scale synthesis of the „semi-final" product Cl-*Lx*-pip-AF (3d)



Supplementary Scheme 11: Synthetic route towards Cl–*Lx*–pip-AF (**3d**) initially used for the lab-scale preparation.[1]

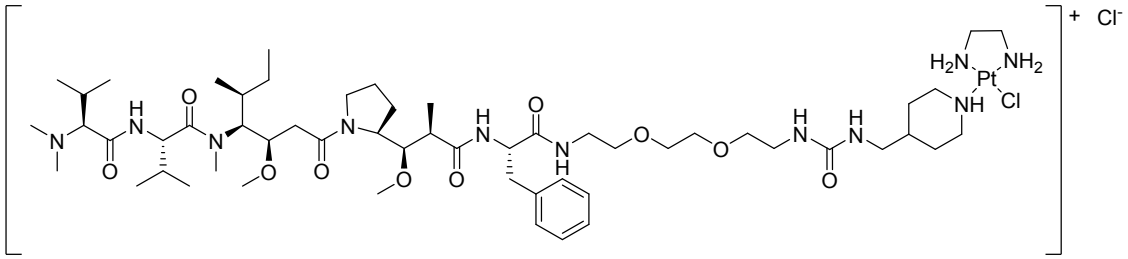
4.1. The laboratory scale synthesis of (*S*)-*N*-((3*R*,4*S*,5*S*)-1-((*S*)-2-((15*S*,18*R*,19*R*)-15-benzyl-18-methyl-3,14,17-trioxo-1-(piperidin-4-yl)-7,10,20-trioxa-2,4,13,16-tetraazahenicosan-19-yl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)-2-((*S*)-2-(dimethylamino)-3-methylbutanamido)-*N*,3-dimethylbutanamide (AF-pip, **1b**)[1]



1b

The synthesis is described in N. J. Sijbrandi *et al.*, *Cancer Res.* **2017**, *77*, 257-267.[1]

4.2. The laboratory scale synthesis of Cl–*Lx*–pip-AF (**3d**)[1]



3d

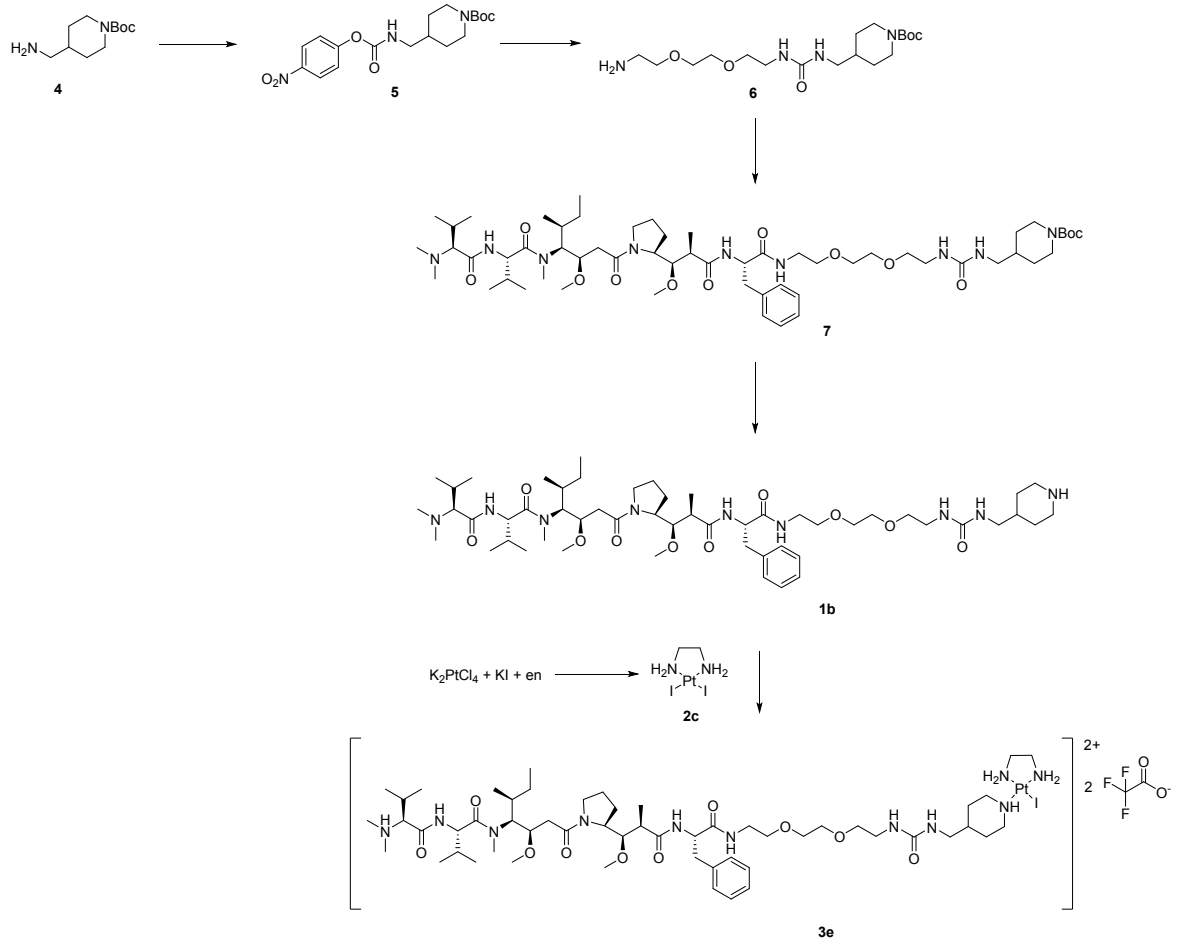
$AgNO_3$ (14.3 mg, 84 μmol, 1.0 eq.) was dissolved in DMF (2 mL) and this solution was added to a suspension of Pt(ethane-1,2-diamine)Cl_2 (**2a**) (*Lx*Cl_2; 50.0 mg, 153 μmol, 1.8 eq.) in DMF (7.5 mL) and stirred for 24 h at room temperature, after which the reaction mixture was filtered through a pad of Celite®.

Subsequently, this solution (3.52 mL, 31 µmol, 1.0 eq.) was added to a solution of *N*-(3-oxo-1-(piperidin-4-yl)-7,10-dioxa-2,4-diazadodecan-12-yl) AF amide (**1b**) (AF-pip; 30.5 mg, 30 µmol, 1.0 eq.) in DMF (1 mL), and the reaction mixture was stirred for 16 h at room temperature in the dark, after which a 20 mM NaCl solution (2 mL) was added, followed by removal of the solvents under reduced pressure. The product was purified by preparative reverse-phase HPLC (10-25% eluent B in eluent A in 40 min.; eluent A: 20 mM NaCl in MiliQ/0.1% TFA, eluent B: 9:1 MeCN/20 mM NaCl in MiliQ/0.1% TFA) and the product fraction (~20 mL) was concentrated to ~4 mL. Subsequently, the solution was diluted with 20 mM NaCl to ~20 mL and loaded on two Sep-Pak® C18 Plus columns in series that had been pre-activated with methanol (20 mL) and water (120 mL). After loading the product, the columns were washed with water (50 mL), purged with air, and the product was eluted with methanol (10 mL). The filtrate was directly concentrated by rotary evaporation, and the trace solvent was removed in high vacuum affording a colorless amorphous solid (21 mg, 52%). The product was stored as a 5 mM solution in 20 mM NaCl/10% DMA at -18 °C.

HPLC analysis showed that the purity of the product was 96%.

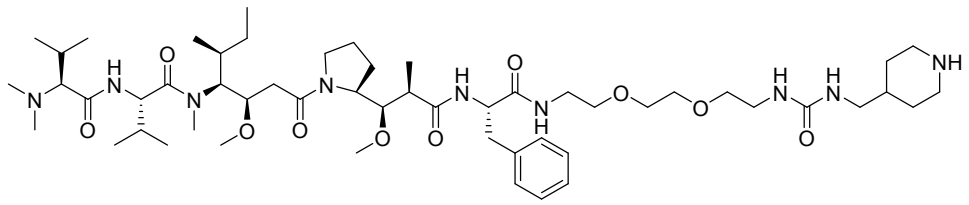
HRMS (ESI$^+$): $C_{55}H_{101}{}^{35}ClN_{11}O_{10}{}^{195}Pt$ $[M]^+$ calc 1305.7069, found 1305.6978.

5. Laboratory scale synthesis of the „semi-final" product I-*Lx*-pip-AF (3e)



Supplementary Scheme 12: Synthetic route towards I–*Lx*–pip-AF (**3e**) used for the laboratory scale preparation.

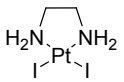
5.1. The laboratory scale synthesis of (*S*)-*N*-((3*R*,4*S*,5*S*)-1-((*S*)-2-((15*S*,18*R*,19*R*)-15-benzyl-18-methyl-3,14,17-trioxo-1-(piperidin-4-yl)-7,10,20-trioxa-2,4,13,16-tetraazahenicosan-19-yl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)-2-((*S*)-2-(dimethylamino)-3-methylbutanamido)-*N*,3-dimethylbutanamide (AF-pip, **1b**)[1]



1b

The synthesis was essentialy the same as described in N. J. Sijbrandi *et al.*, *Cancer Res.* **2017**, *77*, 257-267.[1]

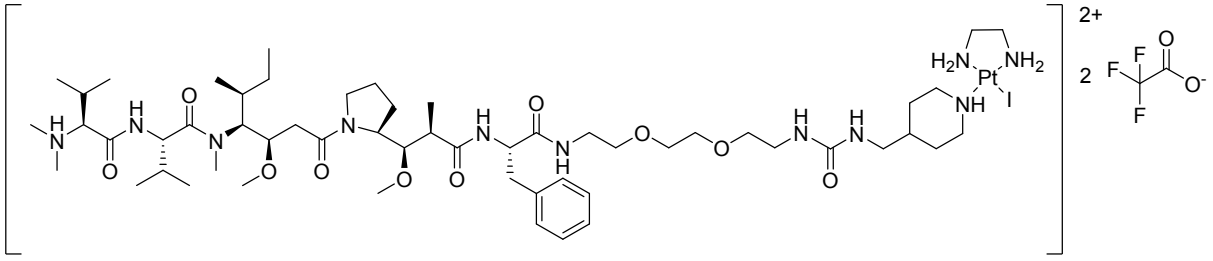
5.2. The laboratory scale synthesis of Pt(ethane-1,2-diamine)I_2 (*Lx*I_2, **2c**)



2c

The synthesis is described in section 2.2.

5.3. The laboratory scale synthesis of I–*Lx*–pip-AF (**3e**) × 2 TFA



3e

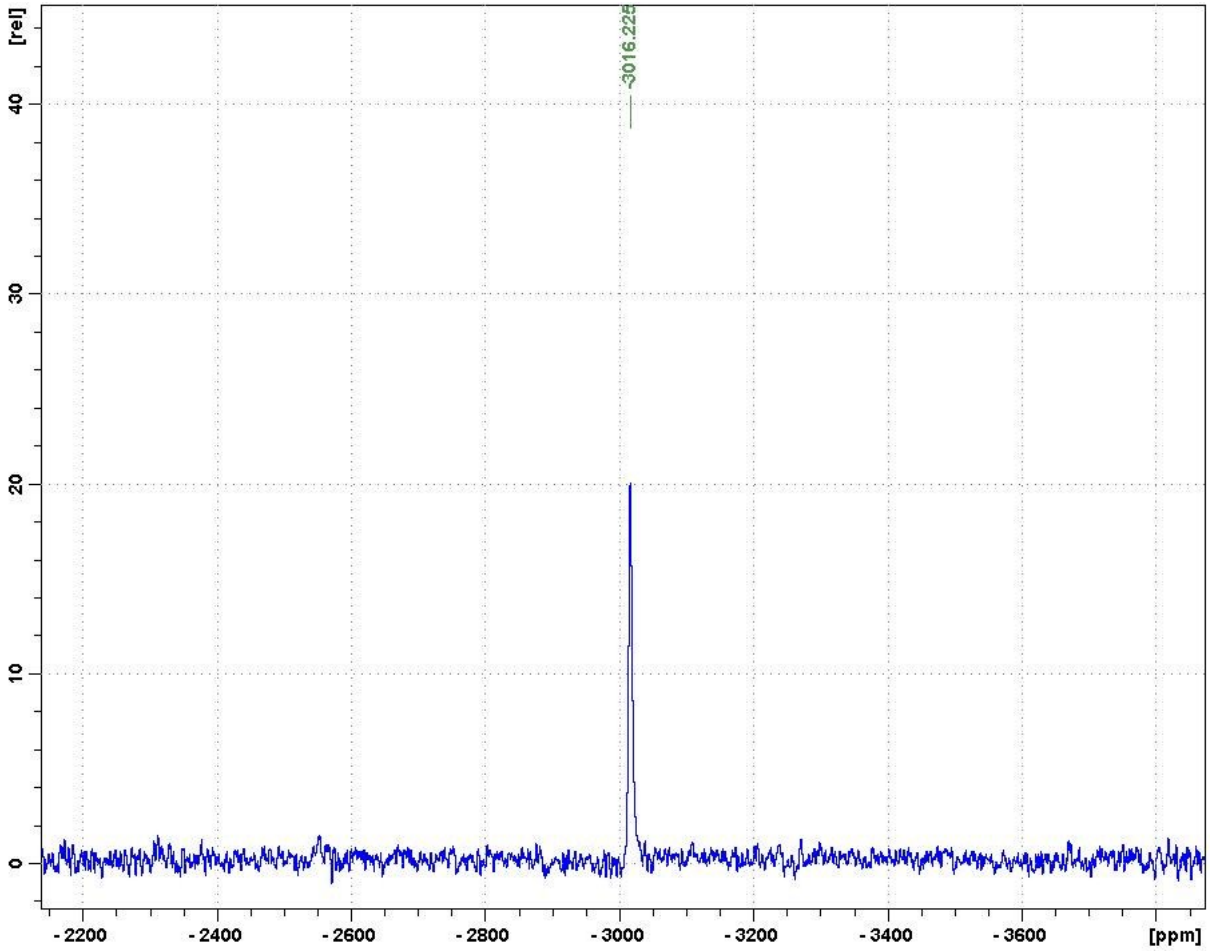
N-(3-Oxo-1-(piperidin-4-yl)-7,10-dioxa-2,4-diazadodecan-12-yl) AF amide (**1b**) (AF-pip; 15.0 mg, 15 µmol, 1.0 eq.) and Pt(ethane-1,2-diamine)I_2 (**2c**) (*Lx*I_2; 22.5 mg, 44 µmol, 3.0 eq.) were dissolved in dry DMF (150 µL) under argon atmosphere. Triethylamine (7.86 µL, 44 µmol, 3.0 eq.) was added and the course of the reaction was followed by HPLC. The reaction mixture was stirred at 60 °C for 2 h. At this moment, the reaction mixture contained 100% product.

The reaction mixture was diluted with water/MeOH (2:1, 2.5 mL) and filtered through a 0.2 µm syringe filter. Purification was performed by preparative reverse-phase HPLC (Grace Alltima C18 5 µm column, 22 × 250 mm; gradient: 35-100% MeOH/0.1% TFA in water/0.1% TFA in 36 min.). Product fractions were concentrated resulting in a colorless oil (18.0 mg, 75% yield; ~15% total yield over 5 linear steps).

HPLC (Grace Alltima C18 5 µm column, 25 × 4.6 mm) indicated that the product was 98.9% pure (retention time t_R = 10.3 min.; gradient: 20-100% MeCN/0.1% TFA in water/0.1% TFA in 20 min., measured at a wavelength of 210 nm).

HRMS (ESI+): $C_{55}H_{102}IN_{11}O_{10}{}^{195}Pt$ $[M+H]^{2+}$ calc 699.3247, found 699.3198.

^{195}Pt-NMR (86 MHz, DMF-d_7): δ -3016.

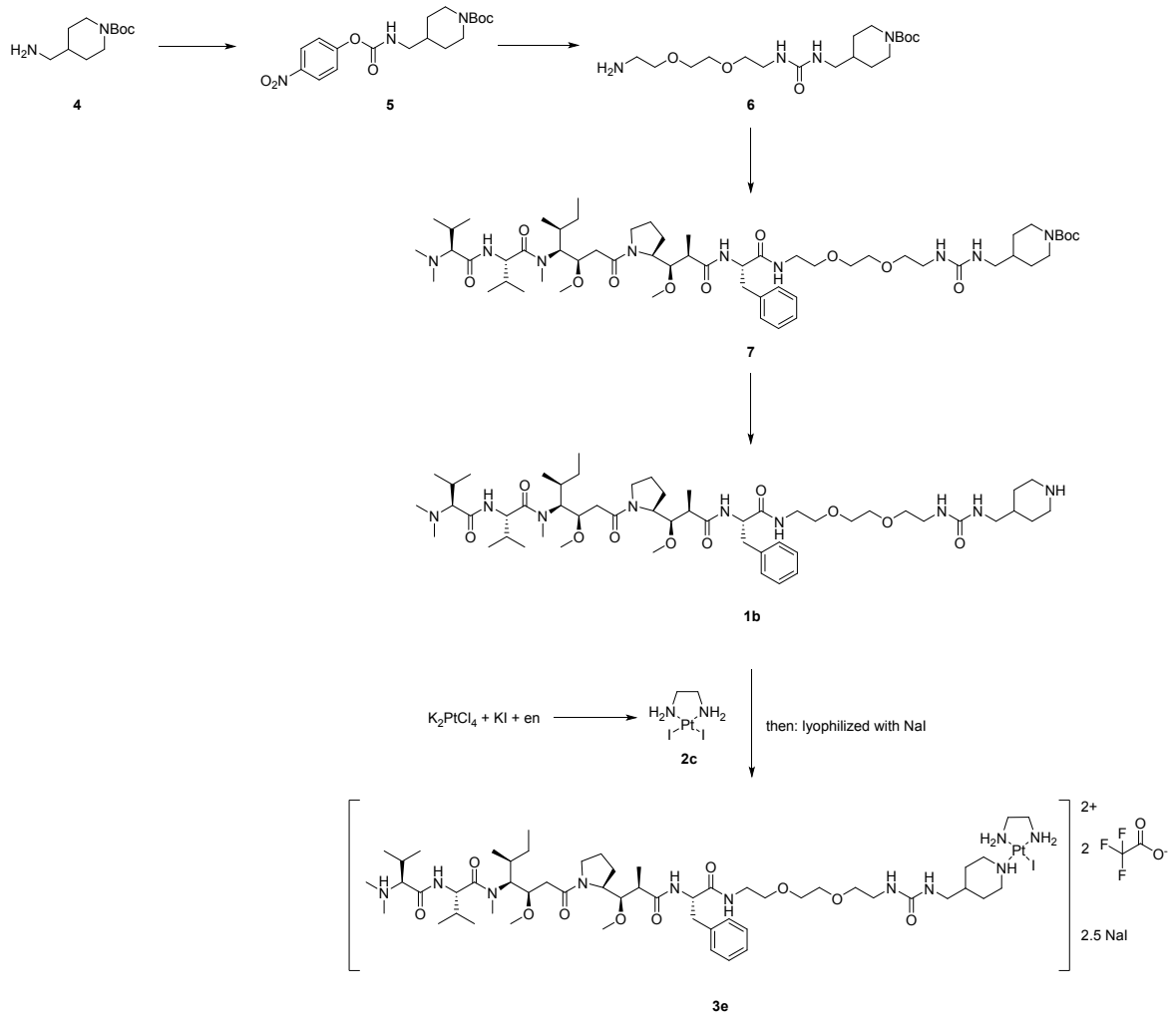


^{195}Pt-NMR spectrum of I–*Lx*–pip-AF (**3e**) × 2 TFA

12.7 mg/0.6 mL DMF-d_7, T = 19.8 °C, number of scans: 500000

Note: the product should be protected from light. Recommended storage temperature: -20 °C.

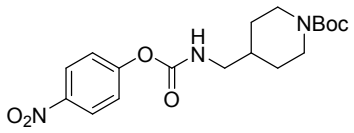
6. Multigram scale synthesis of the „semi-final" product I–*Lx*–pip-AF (3e)



Supplementary Scheme 13: Synthetic route towards the lyophilizate I–*Lx*–pip-AF (**3e**) × 2.0 TFA × 2.5 NaI used for the multigram-scale manufacturing.[1]

The synthesis was optimized in regard to the manufacturing on scale, as outlined below.

6.1. Synthesis and analytical characterization of *tert*-butyl 4-((((4-nitrophenoxy)carbonyl)amino)methyl)piperidine-1-carboxylate (**5**)



5

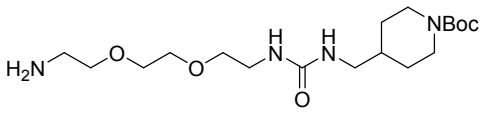
4-Nitrophenyl carbonochloridate (28.8 g, 142.8 mmol, 1.0 eq.) was added as a solid to a solution of *tert*-butyl 4-(aminomethyl)piperidine-1-carboxylate (**4**) (30.6 g, 142.8 mmol, 1.0 eq.) and pyridine (11.5 mL, 142.8 mmol, 1.0 eq.) in DCM (710 mL) under nitrogen atmosphere at room temperature. An exothermic reaction was observed during the addition, whereby the temperature increased temporarily to 30 °C and was allowed to cool again to room temperature; the addition rate of 4-nitrophenyl carbonochloridate was therefore lowered. The solution was clear and turned from light yellow to light orange.

The reaction was stirred overnight at room temperature. In-process control (IPC) was performed by TLC in ethyl acetate/hexanes (1:1) and the reaction showed a full conversion of the starting material after 26 h.

After that, the reaction mixture was washed with sat. aq. $NaHCO_3$ (2 × 400 mL), water (400 mL; a turbid aqueous layer was observed and ~15 min. were required for the layers to separate fully), and brine (400 mL). The organic layer was dried with $MgSO_4$, filtered, and concentrated under reduced pressure to yield a yellow oil.

93.1 g of the crude yellow oil were dissolved in toluene (716 mL; 7.7 × crude weight) at 80 °C and allowed to cool to room temperature slowly overnight. Then, the mixture was first put on ice and then cooled further to -40 °C. Crystals were filtered and dried overnight at 40 °C under vacuum (34.1 g, 63% yield).

6.2. Synthesis and analytical characterization of *tert*-butyl 4-(12-amino-3-oxo-7,10-dioxa-2,4-diazadodecyl)piperidine-1-carboxylate (**6**)



6

Triethylamine (12.6 mL, 89.6 mmol, 1.0 eq.) and 2,2'-(ethane-1,2-diylbis(oxy))diethanamine (65.4 mL, 66.4 g, 448 mmol, 5.0 eq.) were dissolved in DCM (900 mL) under nitrogen atmosphere.

tert-Butyl 4-((((4-nitrophenoxy)carbonyl)amino)methyl)piperidine-1-carboxylate (**5**) (34.0 g, 89.6 mmol, 1.0 eq.) was dissolved in DCM (500 mL) and the solution was added dropwise to the above mixture over 1 h (only 1-2 °C temperature increase was observed), after which the reaction mixture was stirred at room temperature. The mixture turned bright yellow and TLC (DCM:MeOH 95:5) indicated a full conversion after 1 h 45 min., followed by TLC.

Then, the reaction mixture was diluted with DCM (900 mL) and washed with 1 M aqueous NaOH (3 × 460 mL) and water (2 × 460 mL). The mixtures were vigorously stirred in a 5 L flask to ensure a good mixing and poured into a 10 L separatory funnel to settle and separate the layers. Then, the product was extracted from the DCM layer with 0.5 M HCl (1 × 900 mL and 1 × 250 mL). The acidic layers were combined and washed with DCM (500 mL). LC showed that no product was present in the DCM layer. The combined aqueous layers were then washed with 2-methyl tetrahydrofuran (500 mL) to remove traces of DCM, whereby LC showed that no product was present in the organic phase. A new portion of 2-methyl tetrahydrofuran (1500 mL) was added to the acidic layer, which was then basified with 5 M NaOH (150 mL) to a pH of >12 (determined to be ~pH 13 by means of the pH paper).

The layers were thoroughly mixed before separation. The basic layer was washed again with 2-methyl tetrahydrofuran (2 × 500 mL, 1 × 200 mL, and 2 × 300 mL). LC showed that some product remained in the basic layer. After this, another 20 mL of 5 M NaOH were added (pH was 14) and the basic layer was washed with 2-methyl tetrahydrofuran (2 × 300 mL), followed by washing with DCM (2 × 300 mL). The DCM layer was not mixed with the 2-methyl tetrahydrofuran layer. Both the 2-MeTHF phase and the DCM phase were separately dried with $MgSO_4$, filtered, and concentrated under reduced pressure, yielding a light yellow oil (22.2 g, 64% yield; four batches were isolated: 7.01 g, 9.43 g, 2.52 g, and 3.21 g).

Three batches (19.6 g, ~48 mmol) were additionally purified by column chromatography. To this end, a silica slurry (200 g, crude mixture to silica ratio ~1:10) was prepared in DCM, poured into a column tube, and was allowed to properly pack. The silica column was topped off with a thin layer of sand to protect the column bed.

The product batches were dissolved in a minimal amount of DCM (25 mL), applied onto the column, and allowed to flow into the silica. Two portions of DCM (2 × 10 mL) were used to rinse the flask and were applied to the column to further move the crude mixture into the silica bed.

The column was then eluted with:

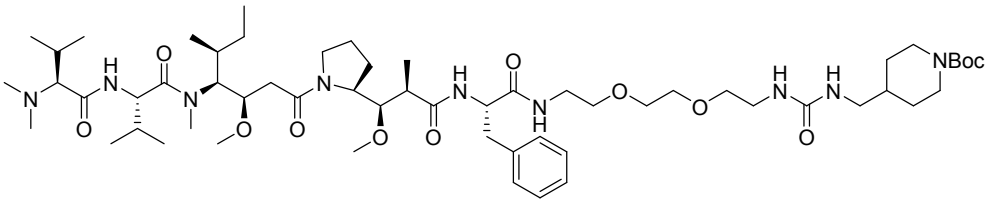
1. 2% methanol in DCM (2 L, ~5 CVs), in order to elute the less polar impurities. Fractions were collected into 500 mL Erlenmeyer flasks.
2. 4% methanol in DCM (1 L, ~2.5 CVs), fractions were collected into 50 mL tubes.
3. 8% methanol with 1% $NH_{3(aq.)}$ in DCM (3.6 L, ~8 CVs) eluting the product. Fractions were collected into 50 mL tubes.

The product containing fractions were evaporated, co-evaporated once with methanol, and dried under high vacuum resulting in a first product batch (10.0 g, 51% yield). Additionally,

further product containing fractions were isolated, co-evaporated with methanol, and dried under vacuum resulting in a second product batch (0.55 g, 3% yield).

A considerable part of the early product containing fractions contained a minor impurity (~1.8 g, ~9% yield) and were therefore not collected.

6.3. Synthesis and analytical characterization of *tert*-butyl 4-((15*S*,18*R*,19*R*)-15-benzyl-19-((*S*)-1-((3*R*,4*S*,5*S*)-4-((*S*)-2-((*S*)-2-(dimethylamino)-3-methylbutanamido)-*N*,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-18-methyl-3,14,17-trioxo-7,10,20-trioxa-2,4,13,16-tetraazahenicosyl)piperidine-1-carboxylate (AF-pip-Boc, **7**)



7

A solution of auristatin F (13.0 g, 15.1 mmol, 1.0 eq.) in DMF (200 mL) was added to the solution of *tert*-butyl 4-(12-amino-3-oxo-7,10-dioxa-2,4-diazadodecyl)piperidine-1-carboxylate (**6**) (8.81 g, 22.7 mmol, 1.5 eq.) in DMF (130 mL) and the mixture was placed into an ice bath and allowed to cool for 10 min. to reach a temperature of 3-4 °C. A 10 µL sample was taken for an in-process control by HPLC and was diluted with acetonitrile (490 µL).

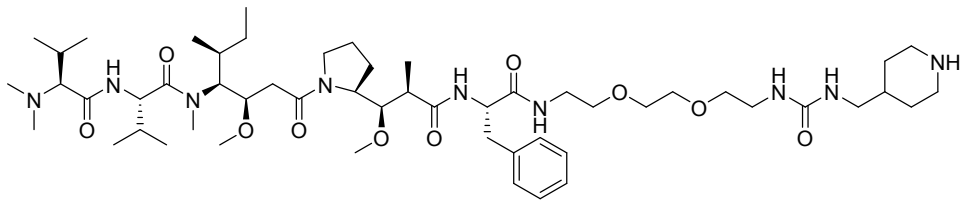
Subsequently, HATU (5.85 g, 15.4 mmol, 1.02 eq.) and DIPEA (7.90 mL, 45.4 mmol, 3.0 eq.) were added and a yellow color developed instantly upon addition of the base. The temperature stayed at 3-4 °C.

The reaction mixture was further cooled and stirred at 0 °C for 20 min.. A 10 µL sample was taken for an in-process control by HPLC and was diluted with acetonitrile (490 µL).

The reaction mixture was evaporated and then co-evaporated twice with toluene (2 × 200 mL) under reduced pressure at 45 °C to remove the major amount of DMF. The crude mixture was then dissolved in DCM (2200 mL) for a further purification.

The reaction mixture was washed with 1 M HCl (3 × 900 mL) and the separated organic phase was washed twice with water (2 × 450 mL). Then, the organic phase was dried with $MgSO_4$ (an in-process control sample was taken for LC) and concentrated under reduced pressure to yield a brittle light brown solid (18.8 g, 112% yield). Considering the DMF content (0.6-0.9 g) and the toluene content (0.3-0.8 g), based on ^1H NMR, the yield was corrected to 17.1-17.8 g (formally 101-106%).

6.4. Synthesis and analytical characterization of (*S*)-*N*-((3*R*,4*S*,5*S*)-1-((*S*)-2-((15*S*,18*R*,19*R*)-15-benzyl-18-methyl-3,14,17-trioxo-1-(piperidin-4-yl)-7,10,20-trioxa-2,4,13,16-tetraazahenicosan-19-yl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)-2-((*S*)-2-(dimethylamino)-3-methylbutanamido)-*N*,3-dimethylbutanamide (AF-pip, **1b**)



1b

AF-pip-Boc (**7**) (18.7 g of material, corresponding to ~17.4 g of compound **7**, ~15.6 mmol, 1.0 eq.) was dissolved in DCM (300 mL). A first in-process control (a 5 µL sample) was taken before adding TFA (300 mL) and stirring the reaction mixture at room temperature. A second in-process control (a 10 µL sample) was taken after 45 min. and showed a full conversion. LC samples were diluted with acetonitrile to a total volume of 500 µL.

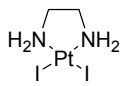
The mixture was evaporated under reduced pressure, after which it was co-evaporated with toluene (100 mL) to remove excess of TFA. During the evaporation of TFA/DCM the collected solvents were pale brown. LC analysis showed that they contained some of the impurity with t_R = 5.6 min., which indicates that this impurity is volatile.

The crude mixture was dissolved in DCM (500 mL). An Isolute SCX-2 column (176 g) was pre-washed with DCM (300 mL), after which the solution of the crude material was applied to the column. The flow through was collected and monitored for unbound material. No unbound material was found.

The column was then washed with DCM (200 mL) and 10% methanol in DCM (700 mL) until no impurities eluted anymore (monitored by LC). Subsequently, the product was eluted using 1 M methanolic ammonia in DCM (1:1). Fractions of 50 mL volume were collected.

All product containing fractions were analyzed by LC, collected and pooled, after which they were evaporated under reduced pressure and co-evaporated with methanol (2 × 75 mL) to remove residual ammonia. The product (15.1 g, 14.9 mmol, 98.5% yield over 2 steps including the HATU coupling) was obtained as an off-white solid.

6.5. Synthesis and analytical characterization of *Lx*I_2 (**2c**)



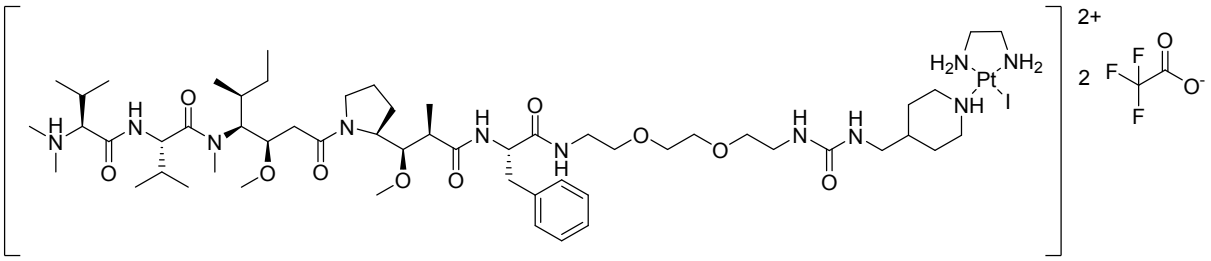
2c

A solution of K_2PtCl_4 (10.0 g, 24.1 mmol, 1.0 eq.) in water (480 mL) was prepared at room temperature. KI (80.0 g, 482 mmol, 20 eq.) was added to the deep red transparent solution, which was then stirred at room temperature for 7.5 h. Upon addition of KI the mixture started to darken. The resulting dark mixture was filtered, ethane-1,2-diamine (1.93 mL, 28.9 mmol, 1.2 eq.) was added, and the mixture was stirred for 16 h at room temperature.

The yellow precipitate that formed was collected by filtration, washed with water, dried on the filter, and subsequently dried in vacuum at 40 °C until a stable weight was obtained, giving a yellow solid (12.0 g, 23.5 mmol, 97.5% yield).

Elemental analysis calc for $C_2H_8I_2N_2Pt$: C, 4.72; H, 1.58; N, 5.50; found: C, 4.73; H, 1.42; N, 5.39.

6.6. Synthesis and analytical characterization of I-*Lx*-pip-AF (**3e**)



3e

N-(3-Oxo-1-(piperidin-4-yl)-7,10-dioxa-2,4-diazadodecan-12-yl) AF amide (**1b**) (AF-pip; 9.5 g, 9.35 mmol, 1.0 eq.) and Pt(ethane-1,2-diamine)I_2 (**2c**) (*Lx*I_2; 14.0 g, 27.4 mmol, 2.9 eq.) were dissolved in DMF (47.5 mL) and kept under the nitrogen atmosphere and in the dark. TEA (3.9 mL, 28 mmol, 3.0 eq.) was added. Based on the TFA content of the starting material **1b** (determined to be 0.01 eq.), no additional TEA was needed. The mixture was stirred at 60 °C until the reaction was finished.

The reaction progress was monitored by HPLC, by taking 5 µL of the reaction mixture and diluting with 495 µL of methanol. The first in-process control (IPC) was performed just after adding TEA. After 1.5 h (second IPC) the reaction was finished and after 2 h the reaction mixture was allowed to cool to room temperature overnight.

Thereafter, the mixture was diluted with water (20 ×, ~900 mL) to precipitate the major amount of unbound *Lx*I_2 (**2c**), and subsequently filtered using filter paper. The filtered *Lx*I_2 (**2c**) was washed with water (100 mL) and dried for 48 h at 40 °C in vacuum. 9.18 g of the dried recovered *Lx*I_2 (**2c**) were obtained (thus, ~4.8 g of *Lx*I_2 (**2c**) were consumed, corresponding to ~9.37 mmol, ~1.0 eq., of *Lx*I_2 (**2c**) that are required stoichiometrically for the reaction).

Afterwards, a short column of 95 g of RP-C18 silica (AF-pip **1b**:silica ratio 1:10) was prepared. The column was prewashed with methanol (3 CVs, ~450 mL) and water/methanol (90:10/0.1% TFA, 3 CVs, ~450 mL). The product mixture was then applied, after which the column was washed with water:methanol (90:10/0.1% TFA, 9 CVs, ~1350 mL) until no more *Lx*I_2 (**2c**) eluted, and with water:methanol (80:20/0.1% TFA, 6 CVs, ~1000 mL) which eluted an impure product containing fraction, after which the main batch of the product was eluted with water:methanol (20:80, 5 CVs, ~750 mL).

The product containing fractions were analyzed on LC, combined, evaporated under reduced pressure, and co-evaporated with methanol (2 × 100 mL) to obtain an off-white to pale yellow solid (7.87 g). The yield was corrected for the TFA content (15 w/w%, which corresponds to 6.69 g of I-*Lx*-pip-AF (**3e**), 4.78 mmol, 51% yield).

The wash fraction containing impure product was also collected, evaporated under reduced pressure, and co-evaporated twice with methanol to obtain a pale brown solid (5.2 g) which was purified additionally as follows. A 52.9 g RP-C18 silica column was prepared, washed with methanol (150 mL, 3 CVs) and water:methanol:TFA (150 mL, 90:10:0.1, 3 CVs). The obtained brown solid was dissolved in methanol (25 mL) and diluted with water (225 mL). The product was applied to the column. Afterwards, the column was washed with water:methanol:TFA (500 mL, 90:10:0.1, 9 CVs) and water:methanol:TFA (350 mL, 80:20:0.1, 6 CVs). Washing was performed until no more *Lx*I_2 (**2c**) eluted from the column. The product was then eluted using water:methanol:TFA (250 mL, 20:80:0.1, 3 CVs). The product fractions were analyzed using LC, combined, evaporated under reduced pressure, and co-evaporated with methanol (2 × 100 mL). This yielded an off-white to pale yellow solid (3.72 g; corrected for the TFA content (16.2 w/w%), it corresponds to 3.12 g of I–*Lx*–pip-AF (**3e**)).

In total, 11.59 g of material were obtained, which corresponds to 9.80 g I–*Lx*–pip-AF (**3e**) (75% yield), obtained as a TFA salt (TFA content: 15.44 w/w%).

6.7. Lyophilization of I–*Lx*–pip-AF (**3e**)

Several batches of I–*Lx*–pip-AF (**3e**) × TFA (18.75 g of material, corresponds to 15.97 g I–*Lx*–pip-AF (**3e**)) of a similar quality were combined and lyophilized.

tert-Butanol was first melted in a stove at 40 °C. The ratio for freezedrying the combined I–*Lx*–pip-AF (**3e**) material was as follows: I–*Lx*–pip-AF (**3e**) × TFA (g) : water (mL) : *tert*-butanol (mL) : NaI (g): 1:15:15:0.225, which required following amounts: 18.75 g (11.4 mmol) of I–*Lx*–pip-AF (**3e**) × TFA, 281.28 g of water, 219.28 g of *tert*-butanol, and 4.27 g of NaI.

NaI (4.27 g) was dissolved in water (281.28 g, concentration: 15 mg/mL).

The I–*Lx*–pip-AF (**3e**) × TFA batches were separately dissolved in *tert*-butanol (total volume of 281.25 mL) and in the aqueous sodium iodide solution (total volume of 281.25 mL) as follows: first, *tert*-butanol was added, in which the material dissolved partially; then, after addition of the sodium iodide solution, the material dissolved fully. The material was dissolved by gentle heating (max. 40 °C) and mixing. Thereafter, the batches were combined and thoroughly mixed using a magnetic stirrer.

The solutions were all yellow, while the solids were of varying degrees of yellowness. Some slight turbidity was observed. No filtration was performed to avoid loss of the possibly not fully dissolved material.

The HDPE amber bottles were flushed with nitrogen and rinsed with Super-Q water (5 mL). The solution was then aliquoted over 30 mL amber bottles and sealed with the corresponding caps. Then, the bottles were frozen in a -40 °C freezer overnight. Frozen mixtures were

visually checked to make sure that there was no liquid still present and the content was properly frozen. In total, 18 bottles were filled (see the table below).

After freezing, 6 bottles were removed from the freezer, the caps were removed, and square tissues were placed over the openings and fastened with elastic bands (preventing direct contact of the solution/compound with the freezedrying setup). The bottles were placed in the freezedryer holders (3 holders, 2 bottles/holder) and connected to the machine, after which the vacuum was switched on. The bottles were left for 2.5-3 d until the holders reached the room temperature. In total, 3 runs were performed to freezedry the whole material.

The product was obtained as a pale yellow powder. The vials were closed with caps, sealed with parafilm, and stored in the freezer at -20 ^{o}C.

The table lists the bottles that were prepared, indicating the mass added to every bottle, the bottle content, and the lost/remaining mass after the lyophilization:

bottle	**added mass (g)**	**I–*Lx*–pip-AF (3e) × TFA × NaI (g)**	**mass lost after drying (g)**	**mass lost after drying (w/w%)**	**mass left in the bottle after drying (g)**	**(mass lost after drying + 4.4) (w/w%)[a,b]**
1	28.0	1.23	26.8	95.8	1.18	100.2
2	28.0	1.23	26.7	95.4	1.28	99.8
3	28.1	1.24	26.9	95.6	1.25	100.0
4	28.1	1.24	26.9	95.6	1.25	100.0
5	28.1	1.24	26.9	95.7	1.22	100.1
6	28.0	1.23	26.8	95.6	1.23	100.0
7	28.0	1.23	26.8	95.5	1.25	99.9
8	28.1	1.24	26.8	95.6	1.24	100.0
9	28.0	1.23	26.8	95.5	1.25	99.9
10	28.0	1.23	26.8	95.6	1.23	100.0
11	27.9	1.23	26.7	95.6	1.23	100.0
12	28.0	1.23	26.8	95.6	1.23	100.0
13	28.0	1.23	26.7	95.6	1.22	100.0
14	28.0	1.23	26.8	95.7	1.22	100.0
15	30.2	1.33	28.9	95.7	1.30	100.1
16	31.8	1.40	30.4	95.6	1.39	100.0
17	31.4	1.38	30.1	95.8	1.32	100.2
18	31.4	1.38	30.0	95.7	1.35	100.1

[a]Based on the weighed amounts in step 2 of this procedure, 523.58 g of the mixture subjected to freezedrying were prepared in total. Of this mixture, 3.581 w/w% was I–*Lx*–pip-AF (**3e**) × TFA and 0.816 w/w% was NaI, which means that 4.397 w/w% was I–*Lx*–pip-AF (**3e**) × TFA × NaI

[b](w/w% of the initial mass added to a bottle that was lost during the freezedrying) + 4.4 w/w% (remaining I–*Lx*–pip-AF (**3e**) × TFA × NaI). The deviation from 100% indicates that not all solvent was removed (<100%), or that more mass was removed than theoretically assumed (>100%). This can be due to weighing or calculation inaccuracies

6.8. Certificate of Analysis (CoA) for I–*Lx*–pip-AF (**3e**) × 2.0 TFA × 2.5 NaI

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CC-CA-2018.112 v03

Title: Certificate of Analysis AF-Lx-I

3. Tests performed for information only

Test	Method	Target values	Result
Appearance	Visual	Report result	Yellow Powder
Identity by ^{1}H NMR spectroscopy	CC-AM-2017.332 v02	Report result	In agreement with reference spectrum
Identity by LC-MS	LC-MS	Report result; Target value $[M+H]^{2+}$ 699.3 m/z	$[M+H]^{2+}$ 699.4 m/z
Identity by ^{195}Pt NMR spectroscopy	NMR	Report result	In agreement with reference spectrum
Content by ^{1}H NMR spectroscopy	CC-AM-2015.674 v01	Report result; Target value 69.4% m/m[3]	67.0% m/m
TFA content by ^{19}F NMR spectroscopy	NMR	Report result; Target value 12.1% m/m[3]	13.2% m/m
Pt content[1]	ICP-OES	Report result; Target value 9.7% m/m[3]	9.4% m/m
Na content[1]	ICP-OES	Report result; Target value 2.8% m/m[3]	2.9% m/m
I Content[1]	Combustion technique	Report result; Target value 22.0% m/m[3]	21.3% m/m
Specified impurities by HPLC - $Pt(en)I_2$ (RRT 0.41) - AF-pip (RRT 0.95)[2]	CC-AM-2018.017 v01	 Report result; Target value < 0.1% m/m Report RRT and % a/a	 ≤ 0.10% m/m 0.24% a/a
Unspecified impurities by HPLC Each ≥ 0.10% a/a - RRT 1.10[2]	CC-AM-2018.017 v01	 Report RRT and % a/a	 0.28% a/a
- Total impurities by HPLC[2]	CC-AM-2018.017 v01	Report result; Target value < 2.0%	0.52% a/a
Residual solvents - Dimethylformamide (^{1}H NMR) - Methanol (GC-HS) - Dichloromethane (GC-HS) - Tert butanol (GC-HS)	CC-AM-2014.034 v04 USP <467>	 Report result (target value < 880 ppm) Report result (target value < 3000 ppm) Report result (target value < 600 ppm) Report result	 8900 ppm <500 ppm <100 ppm 20520 ppm
- Water content	CC-AM-2014.122 v02 USP <921>	Report result	1.8% m/m

[1] Data generated by Solvias AG, Switzerland.

[2] Impurities calculations were corrected for the presence of the lyophilization excipient NaI.

[3] Target values for these contents were based on weighed amounts of material prior to lyophilization.

6.9. Stability data for I–*Lx*–pip-AF (**3e**) × 2.0 TFA × 2.5 NaI at -20 °C

	time point (months)	
	initial	**3 months**
appearance	yellow powder	yellow powder
purity & impurities		
purity (% a/a)	99.5	99.0[a]
impurities ≥ 0.10% a/a:		
RRT 0.41 (*Lx*I_2) (% m/m)[b]	≤ 0.10	< 0.10
RRT 0.87	n.d.	0.68[a]
RRT 0.95-0.96 (AF-pip (**1b**))	0.24	0.33
RRT 1.10	0.28	n.d.
total impurities (% a/a)	0.52	1.00

n.d. = not detected. RRT = relative retention time.

[a] 3 months' time point was measured on a different, more advanced, HPLC system than the initial time point.

[b] for this crucial conjugatable impurity, not % a/a but % m/m was determined.

7. References

1. N. J. Sijbrandi, E. Merkul, J. A. Muns, D. C. J. Waalboer, K. Adamzek, M. Bolijn, V. Montserrat, G. W. Somsen, R. Haselberg, P. J. G. M. Steverink, H.-J. Houthoff and G. A. M. S. van Dongen, *Cancer Res.* **2017**, *77*, 257-267.