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Total synthesis of AsLn2 – a luciferin analogue from the Siberian bioluminescent earthworm *Fridericia heliota*

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GENERAL

NMR spectra of AsLn2 were acquired in D_2O, pH 7.0 (as pH-meter readings) at 30 ^oC on a Bruker Avance III 600 MHz NMR spectrometer equipped with 5 mm CPTXI cryoprobe (Bruker Corporation, Billerica, MA, USA). The following spectra were used to identify the chemical structure of natural and synthetic AsLn2: ^1H (figures S1-S4), ^{13}C (natural only), 2D COSY (figures S1, S2), 2D ^1H-^{13}C HSQC (figures S3, S4), 2D ^1H-^{13}C HMBC (J_{long}=7 Hz) and 2D ^1H-^{15}N HMBC (J_{long}=5 Hz) (natural only). Free Induced Decay (FID) resolution for indirect dimensions was 30 Hz for ^{13}C, 6Hz for ^1H and 95Hz for ^{15}N. Chemical shifts are given in parts per million (ppm). Data acquisition and analysis were performed using Bruker TopSpin software. Chemical shifts of synthetic AsLn2 are identical to the previously published NMR data of natural AsLn2[1] with one assignment error corrected (assignments of carbons 4' and 6' have been redefined). NMR chemical shifts of synthetic AsLn2 are listed in Table S1.

High-resolution mass spectra were obtained on an Agilent 6224 TOF LC/MS System (Agilent Technologies, Santa Clara, CA, USA) equipped with a dual-nebulizer ESI source. Data acquisition and analysis was performed by the MassHunter Workstation software (Agilent Technologies, Santa Clara, CA, USA).

HPLC chiral analysis was performed on Waters Breeze HPLC System equipped with autosampler 2707, a binary HPLC pump 1525, Waters photodiode array detector 2998 and Waters fraction collector III using Ultron ES-OVM chiral column, 150 x 4.6 mm, 5 μm particle size, at 20 °C, UV detection monitored at 254 nm. Mobile phase*:* 20mM potassium phosphate (pH 4.6)/ acetonitrile (HPLC grade) (*85:15*), flow rate 1.0 mL/min.

Table S1 NMR Chemical shifts and proton multiplicities of synthetic AsLn2 in D_2O, pH 7.0 at 30°C. "—" - not applicable. These data are identical to the previously published[1] with one assignment error corrected (assignment of carbons 4' and 6' are redefined).

Fragment and carbon atom number		^{1}H chemical shift and multiplicity	^{13}C chemical shift
Lys	1	—	174.70
	2	3.724 dd(5.6 Hz, 6.8 Hz)	54.77
	3	1.928, 1.875 m, m	30.19
	4	1.463 m	22.02
	5	1.666 quintet (7.2 Hz)	28.21
	6	3.411 t (7.0 Hz)	39.09
CompX	1'	—	165.40
	2'	—	146.09
	3'	6.744 (s)	120.19
	4'	—	123.41
	5'	7.970 (d, 1.7 Hz)	130.66
	6'	—	117.81
	7'	—	160.09
	8'	6.951 (d, 8.7 Hz)	118.54
	9'	7.743 (dd, 1.7 & 8.7 Hz)	134.77
	10'	—	169.41
	11'	3.467 (s)	59.27
Tyr	1"	—	177.79
	2"	4.533 dd(5.1 Hz, 8.2 Hz)	56.24
	3"	3.228 dd(5.1 Hz, 14.1 Hz) 2.973 dd(8.2 Hz, 14.1 Hz)	36.72
	4"	—	129.49
	5",9"	7.144 d(8.4 Hz)	130.59
	6",8"	6.824 d(8.4 Hz)	115.30
	7"	—	154.12

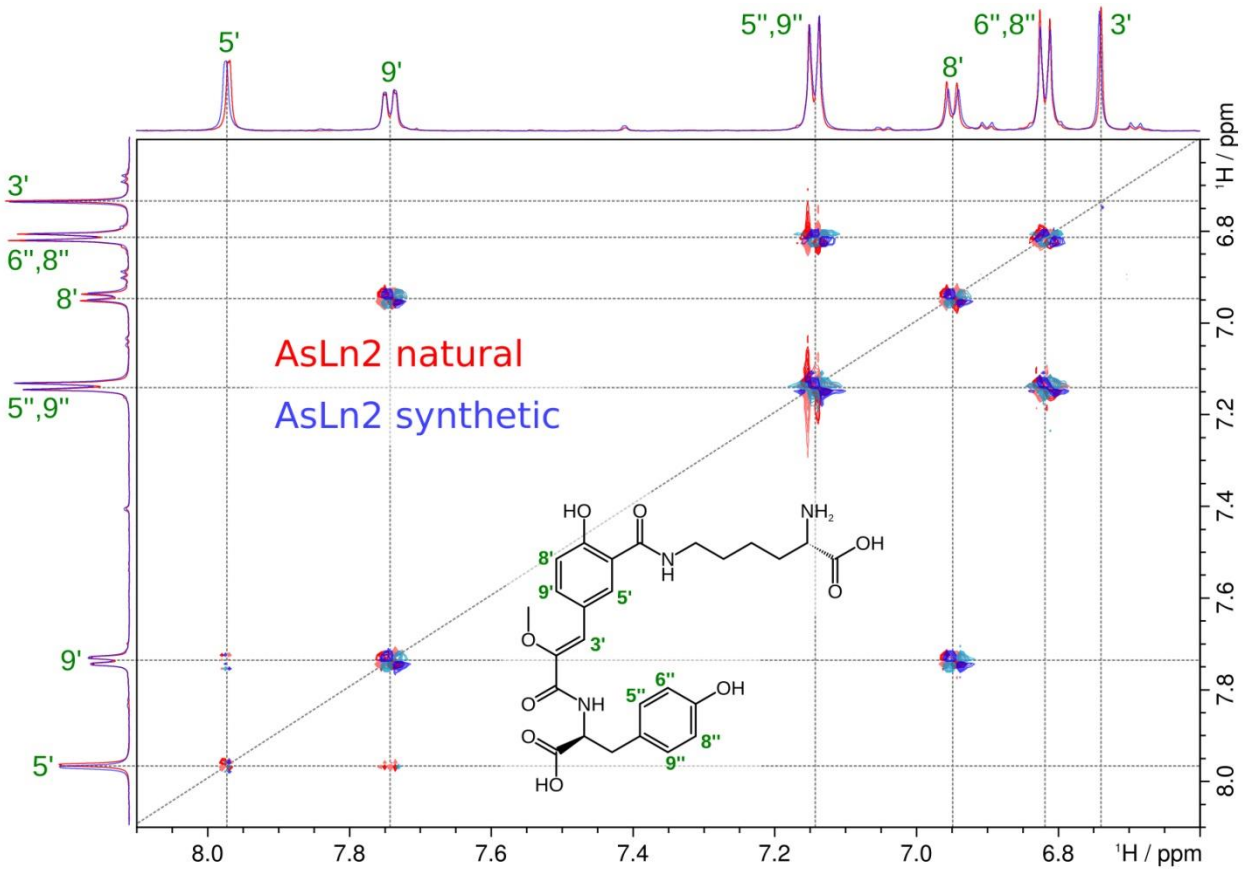

Figure S1 Overlay of ^{1}H and 2D COSY NMR spectra (aromatic region) of synthetic (blue) and natural[1] (red) AsLn2 (D_2O, 30°C, pH 7.0). Signal assignments are labeled.

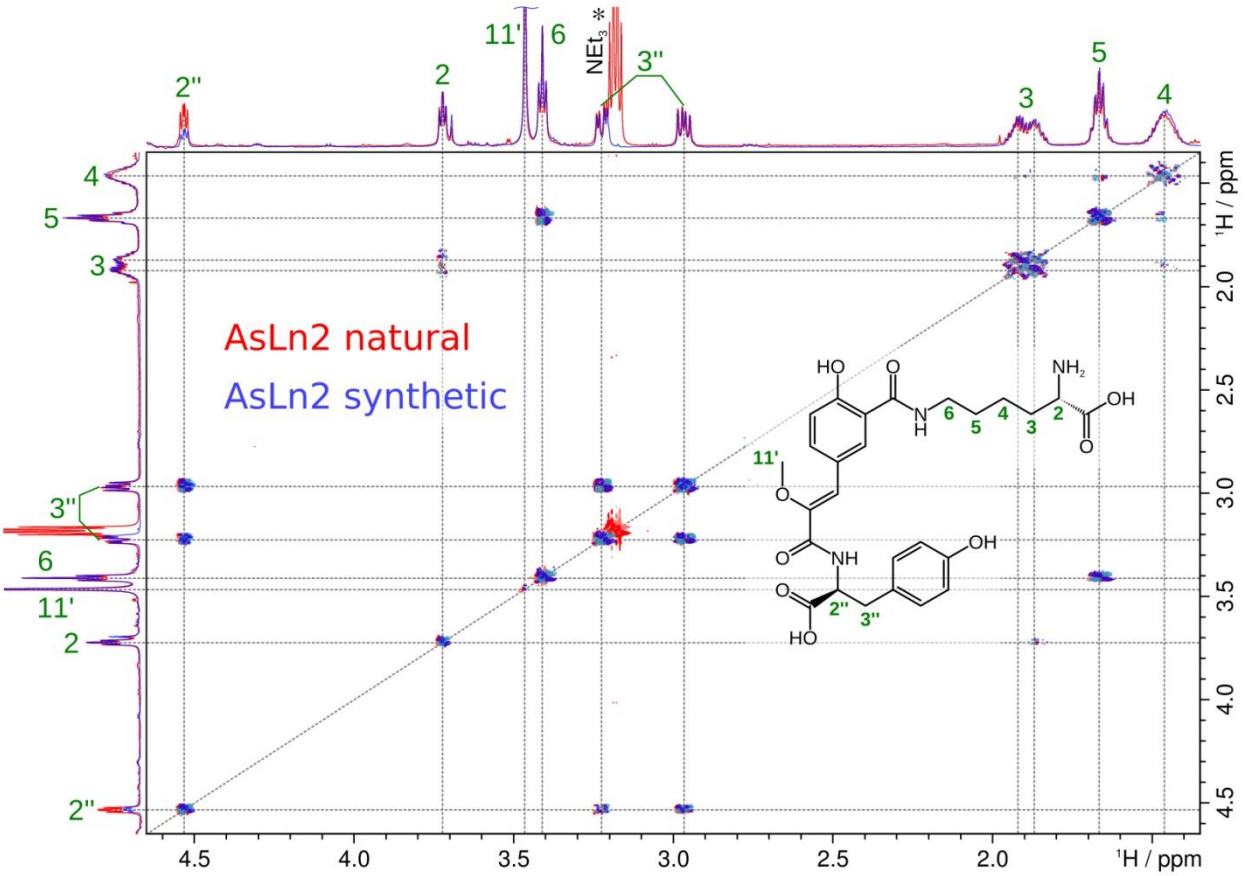

Figure S2 Overlay of ^{1}H and 2D COSY NMR spectra (aliphatic region) of synthetic (blue) and natural[1] (red) AsLn2 (D_2O, 30^{o}C, pH 7.0). Signal assignments are labeled.

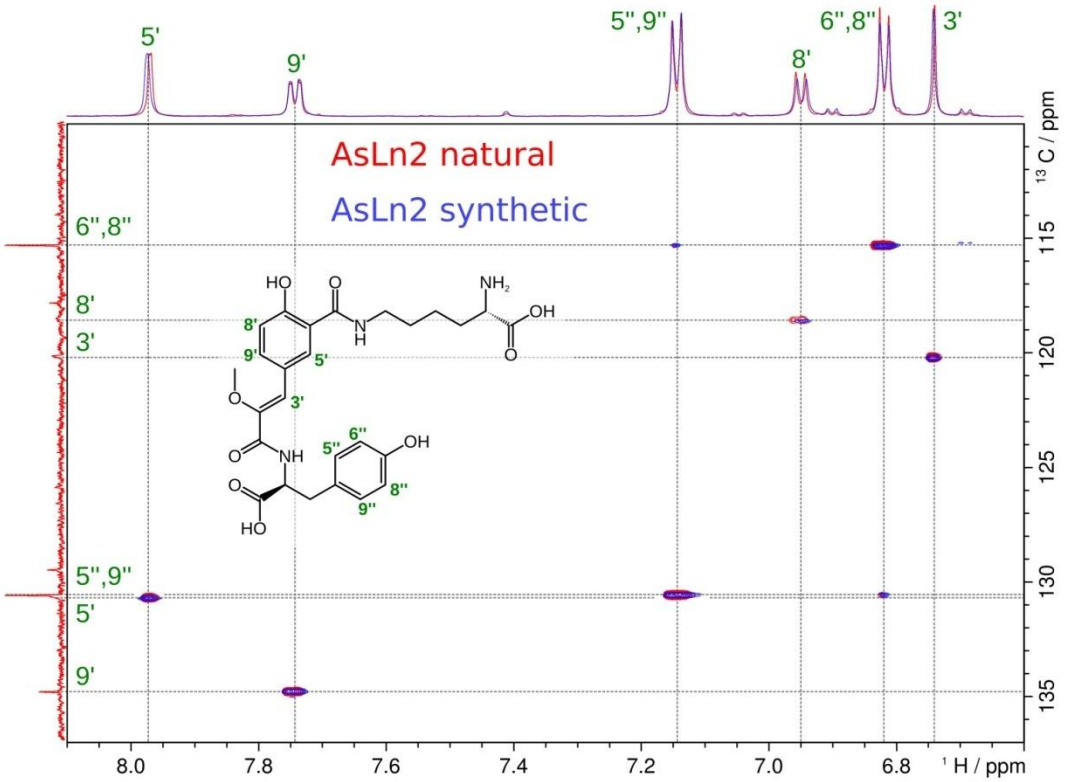

Figure S3 Overlay of ^{1}H and 2D ^{1}H-^{13}C HSQC NMR spectra (aromatic region) of synthetic (blue) and natural[1] (red) AsLn2 (D_2O, 30°C, pH 7.0). Signal assignments are labeled.

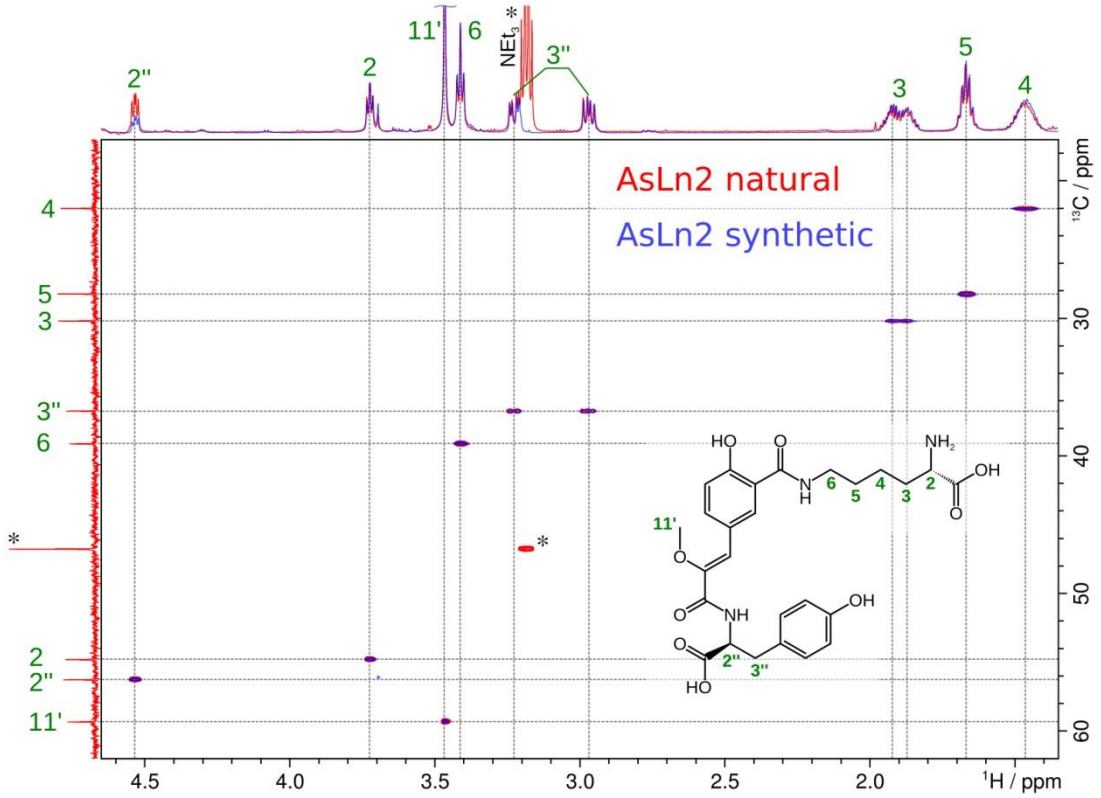

Figure S4 Overlay of ^{1}H and 2D ^{1}H-^{13}C HSQC NMR spectra (aliphatic region) of synthetic (blue) and natural[1] (red) AsLn2 (D_2O, 30°C, pH 7.0). Signal assignments are labeled.

HRMS DATA FOR NATURAL AND SYNTHETIC ASLN2

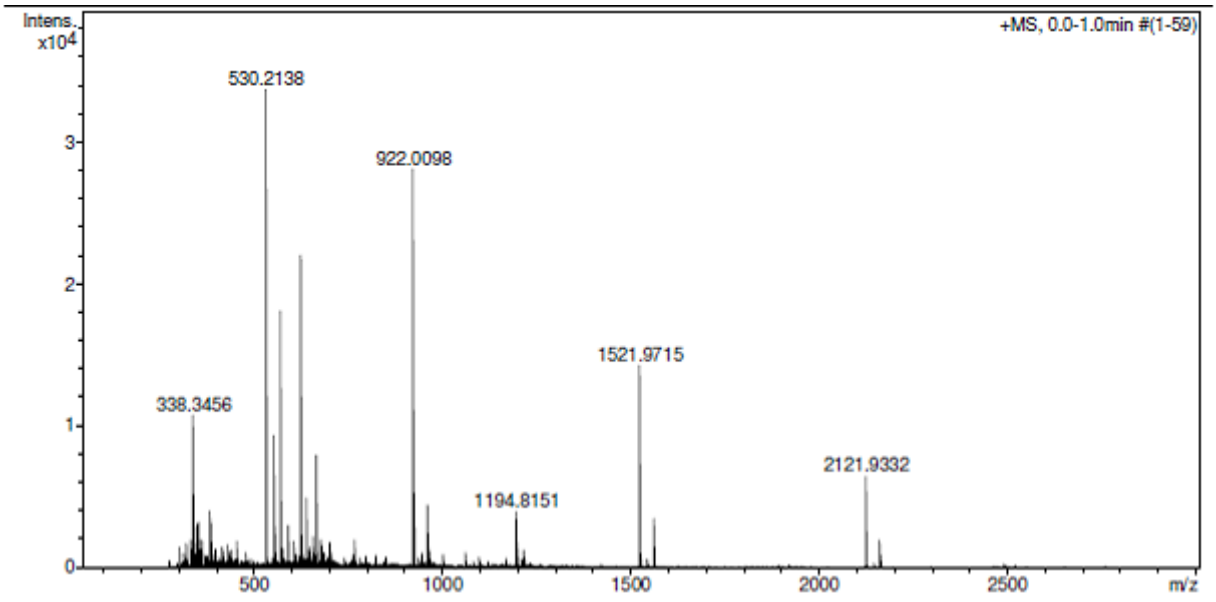

Figure S5 HRMS spectrum of natural AsLn2.

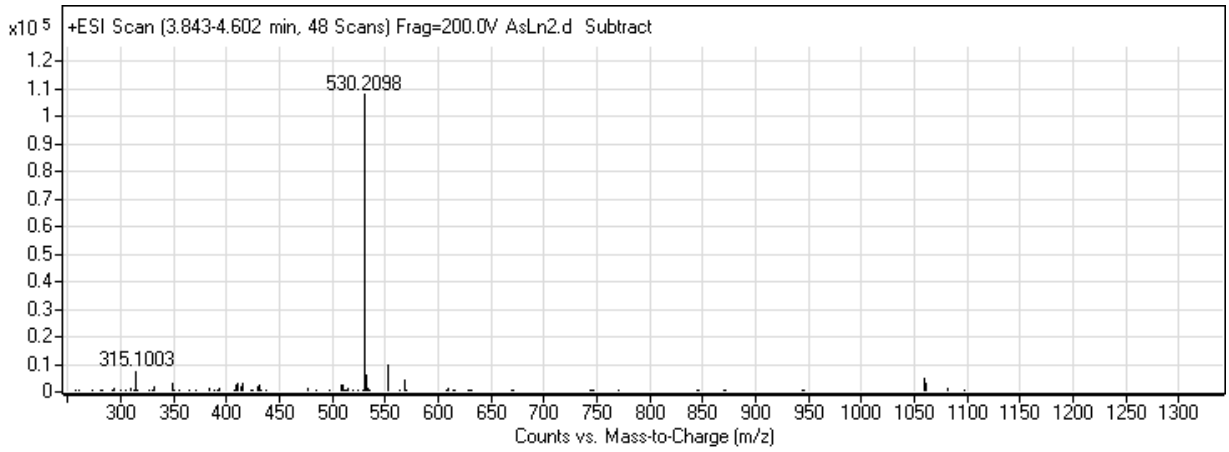

Figure S6 HRMS spectra of synthetic AsLn2.

CHIRAL HPLC DATA

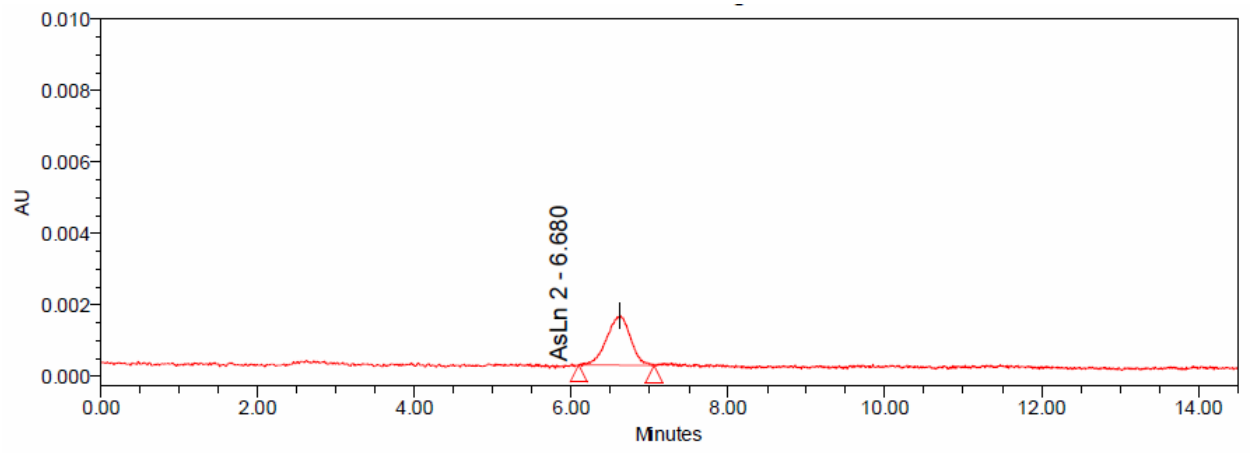

Figure S7 Chromatogram of natural AsLn2 on Ultron ES-OVM chiral column.

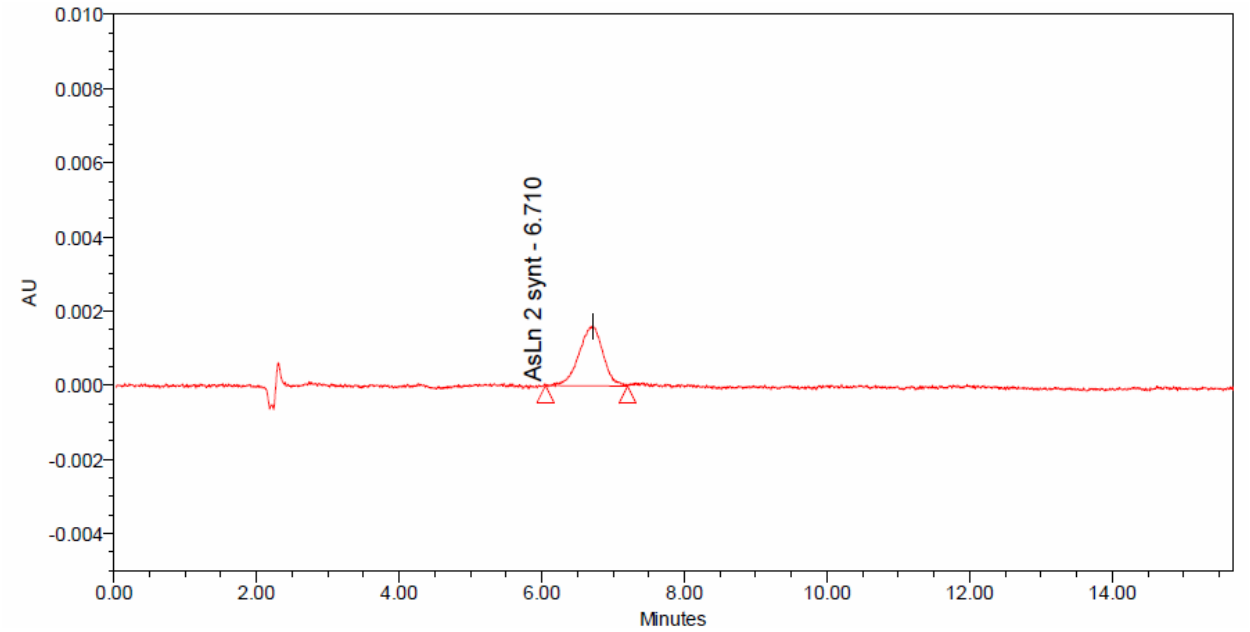

Figure S8 Chromatogram of synthetic AsLn2 on Ultron ES-OVM chiral column.

SYNTHETIC PROCEDURES FOR ASLN2

(*Z*)-2-[(*tert*-Butoxycarbonyl)amino]-6-[5-(2,3-dimethoxy-3-oxoprop-1-en-1-yl)-2-hydroxybenzamido]hexanoic acid (2). A mixture of **1** (CompX-OMe, 0.50 g, 2.0 mmol), *N,N'*-dicyclohexylcarbodiimide (DCC)(0.70 g, 3.4 mmol) and *N*-hydroxysuccinimide (SuOH) (0.46 g, 4.0 mmol) in 30 ml of THF was stirred at 25 °C for 3 h. Upon completion 1.25 g (5.0 mmol) of N_{α}-Boc-L-lysine and 1g (10 mmol) of NEt_3 were added, the mixture was stirred at 25°C for 12 h. The solution was acidified to pH 3.0 with aqueous HCl and extracted with EtOAc (3x150 ml). The extract was dried over Na_2SO_4, evaporated under reduced pressure and the residue subjected to column chromatography on silica gel ($CHCl_3$: MeOH : AcOH, 97:2:1), Rf 0.45, yielding 1.0 g (53%) of **2**, a colorless oil.^{1}H NMR (600 MHz, $CDCl_3$): δ 12.59 (brs, 1H), 7.87 (d, *J* = 1.8 Hz, 1H), 7.79 (dd, J= 8.7, 1.8 Hz,1H), 6.98 (d, *J* = 8.7 Hz, 1H), 6.93 (s, 1H), 6.67 (brs, 1H), 5.17 (brs, 1H), 4.35-4.31 (m, 1H), 3.85 (s, 3H), 3.77 (s, 3H), 3.48-3.44 (m, 2H), 1.95-1.88 (m, 1H), 1.78-1.65 (m, 3H), 1.53-1.48 (m, 2H), 1.43(s, 9H).

(*Z*)-*tert*-Butyl 2-[(*tert*-butoxycarbonyl)amino]-6-[5-(2,3-dimethoxy-3-oxoprop-1-en-1-yl)-2-hydroxybenzamido]hexanoate (3). *N,N'*-diisopropyl-*O*-*tert*-butylisourea (1.7 g, 8.5 mmol) was added to a solution of **2** (0.81 g, 1.7 mmol) in THF (10 ml), the mixture was stirred at 25°C for 14 h. The precipitate of *N,N'*-diisopropylurea was filtered and the solution was dried over Na_2SO_4, evaporated under reduced pressure and the residue was purified by column chromatography on silica gel ($CHCl_3$: MeOH : AcOH, 97:2:1), Rf 0.60, yielding 0.7 g (78%) of **3**, a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ 12.62 (s, 1H), 7.83 (dd, J= 8.7, 1.9 Hz,1H), 7.81 (d, *J* = 1.9 Hz, 2H), 6.98 (d, *J* = 8.7 Hz, 1H), 6.91 (s, 1H), 6.61 (brs, 1H), 5.09 (brs, 1H), 4.14-4.19 (m, 1H), 3.84 (s, 3H), 3.77 (s, 3H), 3.49-3.43 (m, 2H), 1.86-1.80 (m, 1H), 1.72-1.63 (m, 3H), 1.51-1.46 (m, 2H), 1.45(s, 9H), 1.42(s, 9H); ^{13}C NMR (176 MHz, $CDCl_3$): δ 171.8, 169.8, 164.8, 162.3, 155.5, 144.2, 135.7, 127.7, 124.2, 123.3, 118.9, 114.4, 82.0, 79.8, 59.2, 53.8, 52.1, 39.5, 32.8, 28.9, 28.3, 28.0, 22.7.

(*Z*)-3-(3-[(6-(*tert*-Butoxy)-5-[(tert-butoxycarbonyl)amino]-6-oxohexyl)carbamoyl]-4-hydroxyphenyl)-2-methoxyacrylic acid (4). NaOH (33 g,0.83 mmol) in 5 ml of H_2O-EtOH mixture (3:1) was added to 175 mg (0.33 mmol) of **3**, the mixture was stirred at 25°C for 3 h. The solution was acidified to pH 4.0 with aqueous acetic acid and extracted with EtOAc (4x50 ml). Solvent was dried over Na_2SO_4, evaporated under reduced pressure and the residue subjected to column chromatography on silica gel ($CHCl_3$: MeOH : AcOH, 94:5:1), Rf 0.55, yielding 150 mg (88%) of **4**, white crystalline solid; mp 81-85 °C. ^{1}H NMR (600 MHz, DMSO): δ 12.84 (brs, 1H), 8.75 (brt, J 5.4 Hz, 1H), 8.08 (d, *J* = 1.8 Hz, 1H), 7.96 (dd, J= 8.7, 1.8 Hz,1H), 7.05 (brd, J 7.6 Hz, 1H), 6.94 (d, *J* = 8.7 Hz, 1H), 6.85 (s, 1H), 3.78-3.74 (m, 1H), 3.70 (s, 3H),

3.32-3.25 (m, 2H), 1.67-1.61 (m, 1H), 1.60-1.49 (m, 3H), 1.40-1.33 (m, 20H); ^{13}C NMR (176 MHz, DMSO):171.8, 168.5, 164.9, 160.4, 155.5, 144.5, 134.1, 130.1, 124.1, 121.6, 117.8, 115.3, 80.1, 77.9, 58.5, 54.2, 38.7, 30.4, 28.3, 28.1, 27.6, 22.9.

(*Z*)-*tert*-Butyl 2-[(*tert*-butoxycarbonyl)amino]-6-[2-hydroxy-5-(3-[(3-(4-hydroxyphenyl)-1-methoxy-1-oxopropan-2-yl]amino)-2-methoxy-3-oxoprop-1-en-1-yl]benzamido)hexanoate (5). A mixture of **4** (100 mg, 0.2 mmol), *N,N'*-dicyclohexylcarbodiimide (DCC) (70 mg, 0.34 mmol) and *N*-hydroxysuccinimide (SuOH) (46 mg, 0.4 mmol) in 3 ml of THF was stirred at 25°C for 3 h. Upon completion 116 mg (0.5 mmol) of L-tyrosine methyl ester hydrochloride and 100 mg (1 mmol) of NEt_3 were added, the mixture was stirred at 25°C for 12 h. The solution was then acidified to pH 3.0 with aqueous HCl, extracted with EtOAc (3x150 ml), dried over Na_2SO_4, evaporated under reduced pressure and the residue subjected to column chromatography on silica gel ($CHCl_3$: MeOH : AcOH, 94:5:1), Rf 0.55, yielding 80 mg (60%) of **5**, a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 12.59 (s, 1H), 7.69 (s, 2H), 7.00 (d, *J* = 8.5 Hz, 2H), 6.96 (d, *J* = 8.9 Hz, 1H), 6.88 (d, *J* = 8.2 Hz, 1H), 6.80 (d, *J* = 8.5 Hz, 2H), 6.75 (s, 1H), 6.68 (brs, 1H), 5.11 (d, *J* = 7.1 Hz, 1H), 4.91 (dd, *J* = 13.8, 6.0 Hz, 1H), 4.17 (d, *J* = 6.0 Hz, 1H), 3.78 (s, 3H), 3.58 – 3.50 (m, 1H), 3.52 (s, 3H), 3.45 – 3.38 (m, 1H), 3.20 (dd, *J* = 14.2, 5.8 Hz, 1H), 3.09 (dd, *J* = 14.2, 6.2 Hz, 1H), 1.86 – 1.80 (m, 1H), 1.74 – 1.63 (m, 3H), 1.51 – 1.42 (m, 20H).^{13}C NMR (201 MHz, $CDCl_3$) δ 175.3, 172.0, 169.8, 163.6, 161.9, 155.8, 155.3, 147.1, 135.4, 130.5, 127.4, 127.2, 124.0, 119.4, 118.9, 115.7, 114.5, 82.3, 80.2, 59.2, 53.9, 53.1, 52.5, 39.4, 36.9, 32.7, 29.7, 28.6, 28.3, 28.0, 22.8, 20.4.

(*Z*)-2-Amino-6-(2-hydroxy-5-[3-([3-(4-hydroxyphenyl)-1-methoxy-1-oxopropan-2-yl]amino)-2-methoxy-3-oxoprop-1-en-1-yl]benzamido)hexanoic acid (6). 1 ml of HBr (33% in glacial acetic acid) was added to a solution of **5** (80 mg, 0.11 mmol) in glacial acetic acid (3 ml), the mixture was stirred for 6 min at 25 °C, then diluted with diethyl ether (30 ml). The precipitate was washed with Et_2O (5x30 ml) and dried at room temperature under reduced pressure, yielding 65 mg (91%) of **6**, white crystalline solid, used at the next stage without purification.

(*Z*)-2-Amino-6-(5-[3-([1-carboxy-2-(4-hydroxyphenyl)ethyl]amino)-2-methoxy-3-oxoprop-1-en-1-yl]-2-hydroxybenzamido)hexanoic acid (7). NaOH (2M, 4 ml) was added to 65 mg (0.1 mmol) of **6**, the mixture was stirred for 5 min at 25 °C. The solution was then acidified to pH 5.0 with aqueous HCl and extracted with EtOAc (5x25 ml), dried over Na_2SO_4, and solvent evaporated under reduced pressure. The residue was purified using HPLC chromatography (see below), yielding 40 mg (72%) of **AsLn2,** white crystalline solid. ^{1}H and ^{13}C NMR data are listed in Table S1.

HPLC CHROMATOGRAPHIC DATA FOR NATURAL AND SYNTHETIC ASLN2

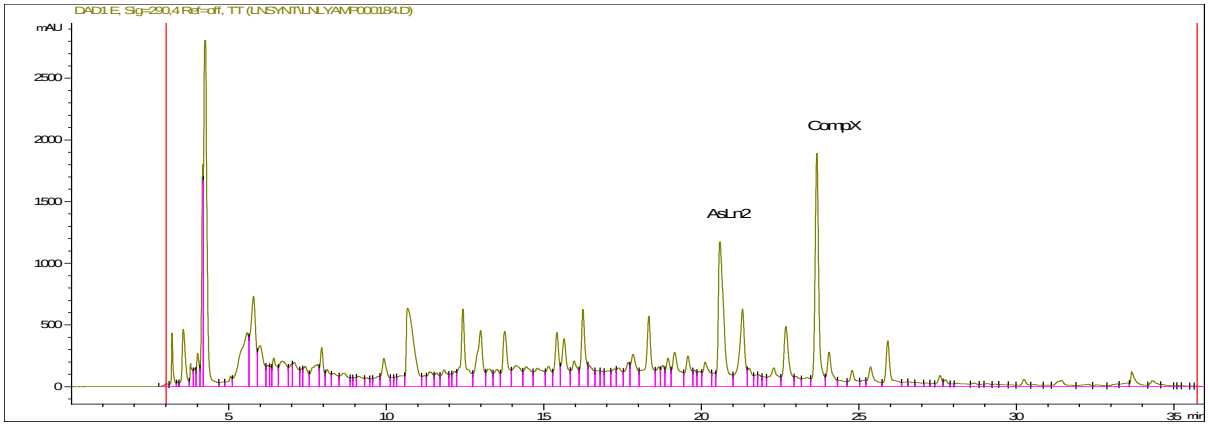

Figure S9 Reverse-phase chromatogram of the fraction (70 mL), containing natural AsLn2 and several other luciferin analogs, using a 9.4 mm x 250 mm ZORBAX Eclipse XDB-C_{18} column.

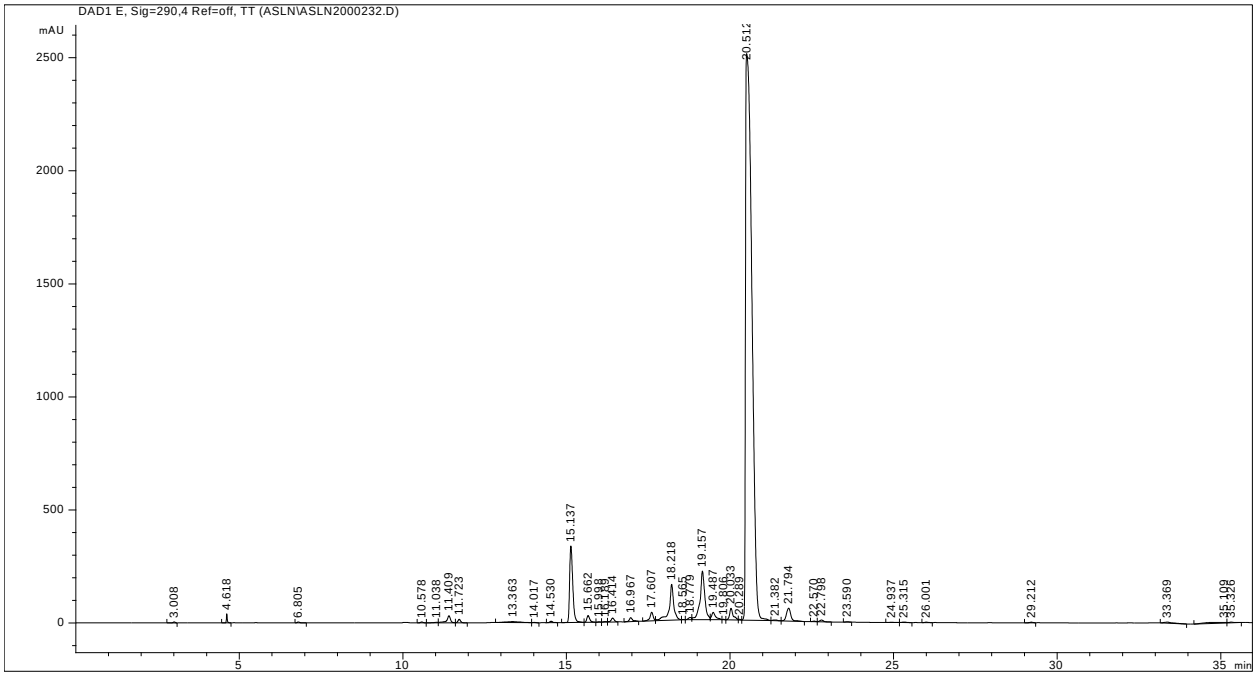

Figure S10 Reverse-phase chromatogram of synthetic AsLn2.

REFERENCES

(1) V. N. Petushkov, M. A. Dubinnyi, N. S. Rodionova, K. D. Nadezhdin, S. M. Marques, J. C. G. Esteves da Silva, O. Shimomura and I. V. Yampolsky, *Tetrahedron Lett.*, 2014, **55**, 463.