Supporting Information

Synthetic and Pre-clinical Characterization of a Cationic Iodinated Imaging Contrast Agent (CA4+) and Its Use for Quantitative Computed Tomography of *Ex Vivo* Human Hip Cartilage

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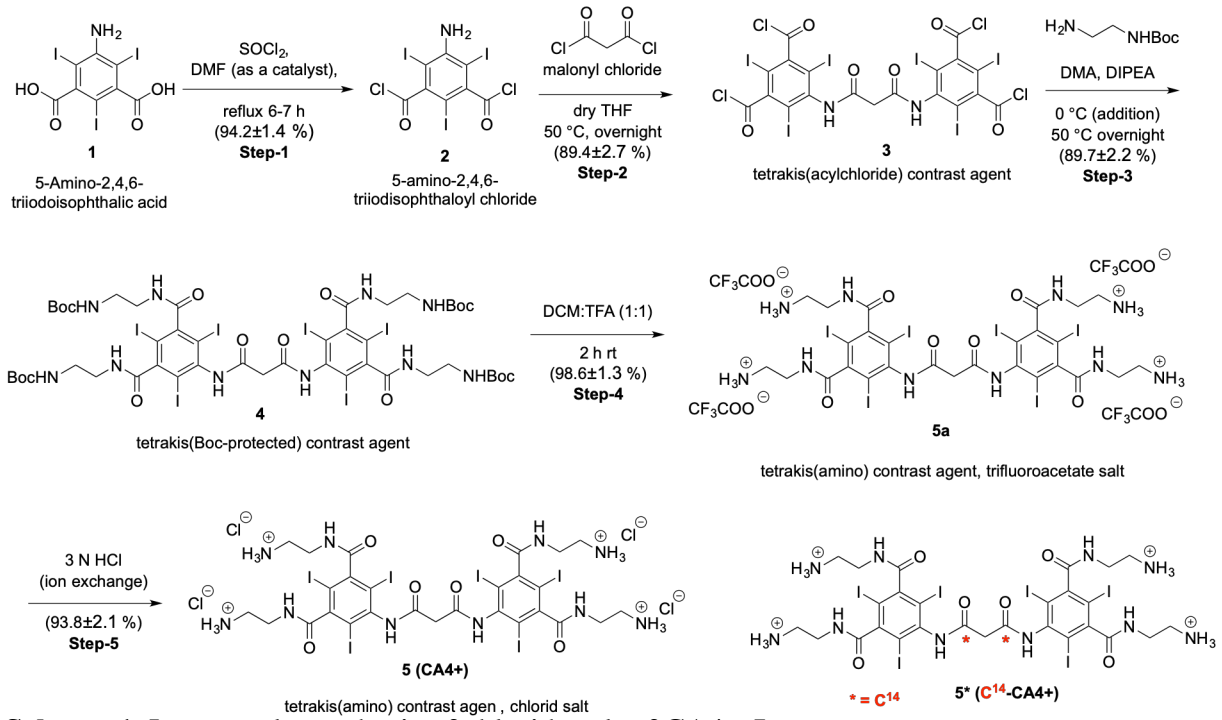

Scheme 1. Large scale synthesis of chloride salt of CA4+ **5**.

Alternative method for the synthesis of 5-amino-2,4,6-triiodoisophthaloyl chloride **2:** Compound **2** was synthesized using ethylene dichloride as a solvent and only four equivalents of thionyl choride. After quenching the thionyl chloride with ice-cold water, the ethylene dichloride layer (highly concentrated) was separated. The aqueous layer was extracted twice with dichloromethane, and the combined organic layers were washed and concentrated to obtain **2** in 96.3 % yield (this reaction was repeated twice). We have successfully synthesized **2** using both procedures and found that the workup is more convenient and simple in the first case (thionyl chloride as a reagent and solvent) than the alternative procedure because of the tedious workup using the chlorinated solvent and poor visual separation of the two layers.

NMR Spectra

Scanned NMR spectra of the synthesized compounds 2, 3, 4, 5a, and 5. All spectra were recorded by Agilent 500 MHz VNMRS spectrometer with a Varian ultra-shielded magnet at 500 MHz for ^{1}H NMR, 470 MHz for ^{19}F NMR, 125 MHz for ^{13}C NMR:

NMR Spectra

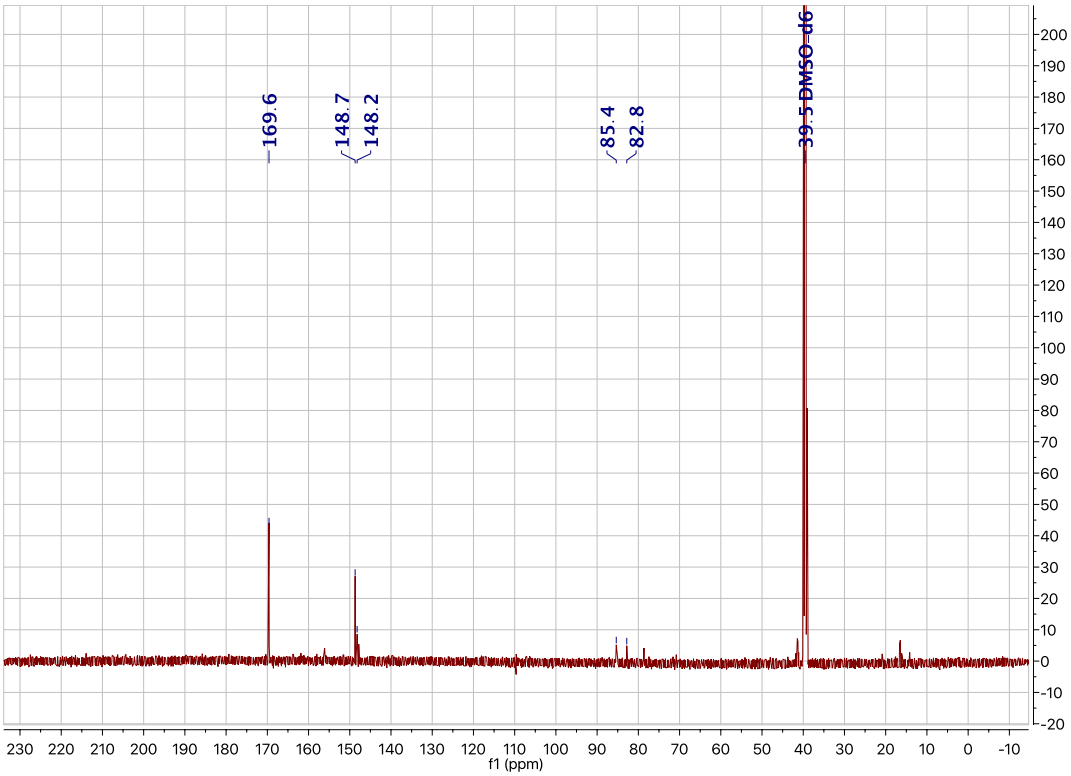

Figure S1. ^{13}C NMR of 5-amino-2,4,6-triiodoisophthaloyl chloride **2**

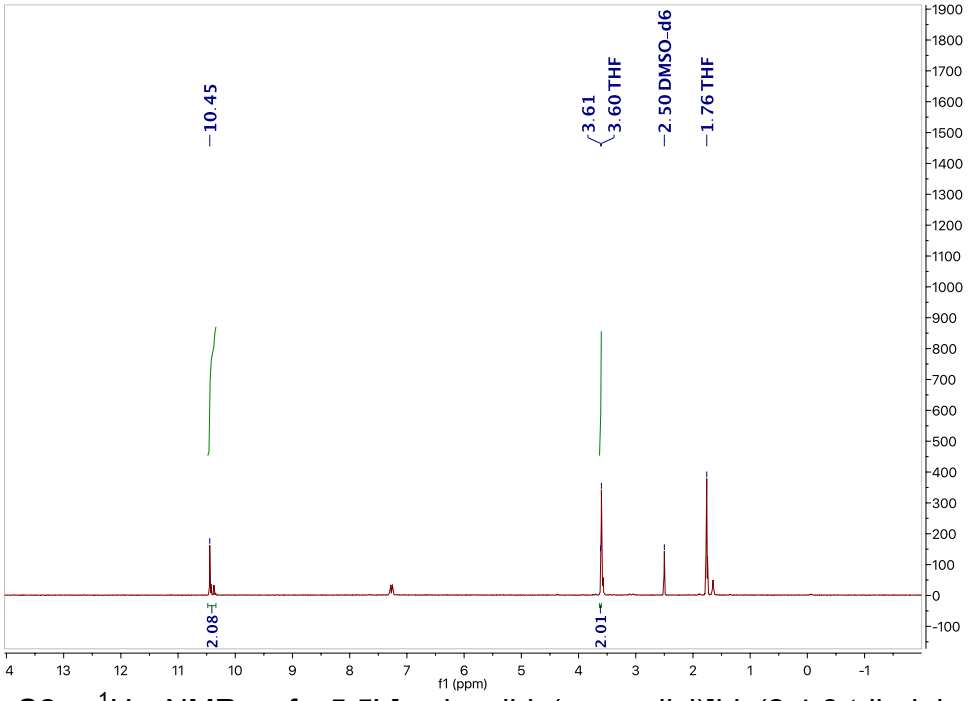

Figure S2. ^{1}H NMR of 5,5'-[malonylbis(azanediyl)]bis(2,4,6-triiodoisophthaloyl dichloride) **3**

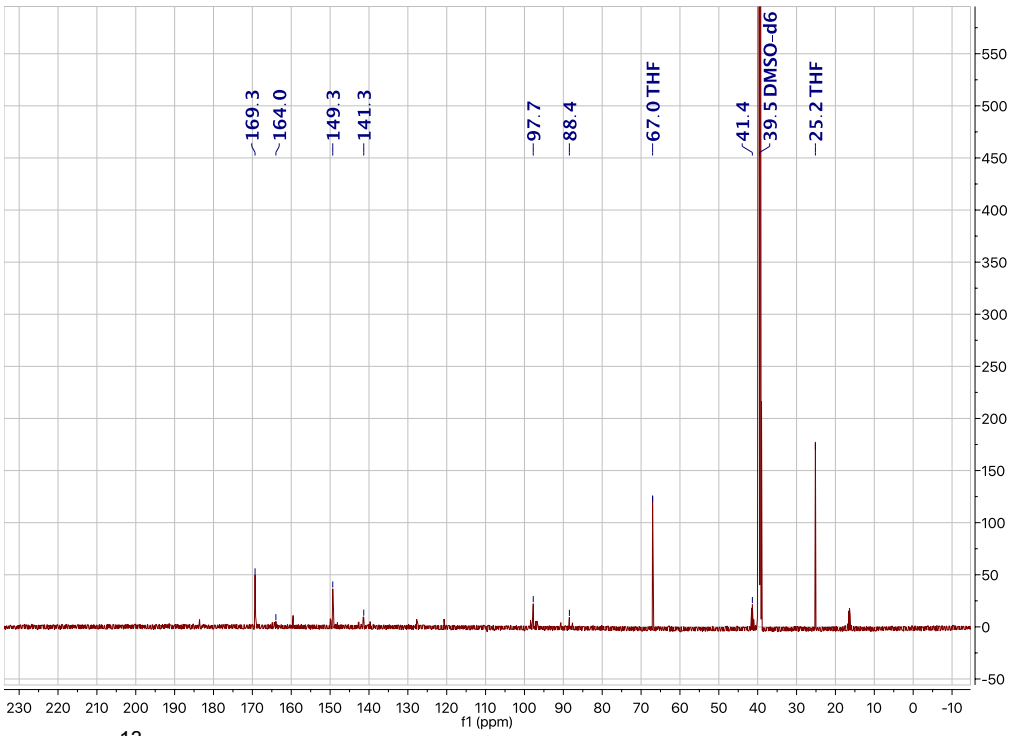

Figure S3. ^{13}C NMR of 5,5'-[malonylbis(azanediyl)]bis(2,4,6-triiodoisophthaloyl dichloride) **3**

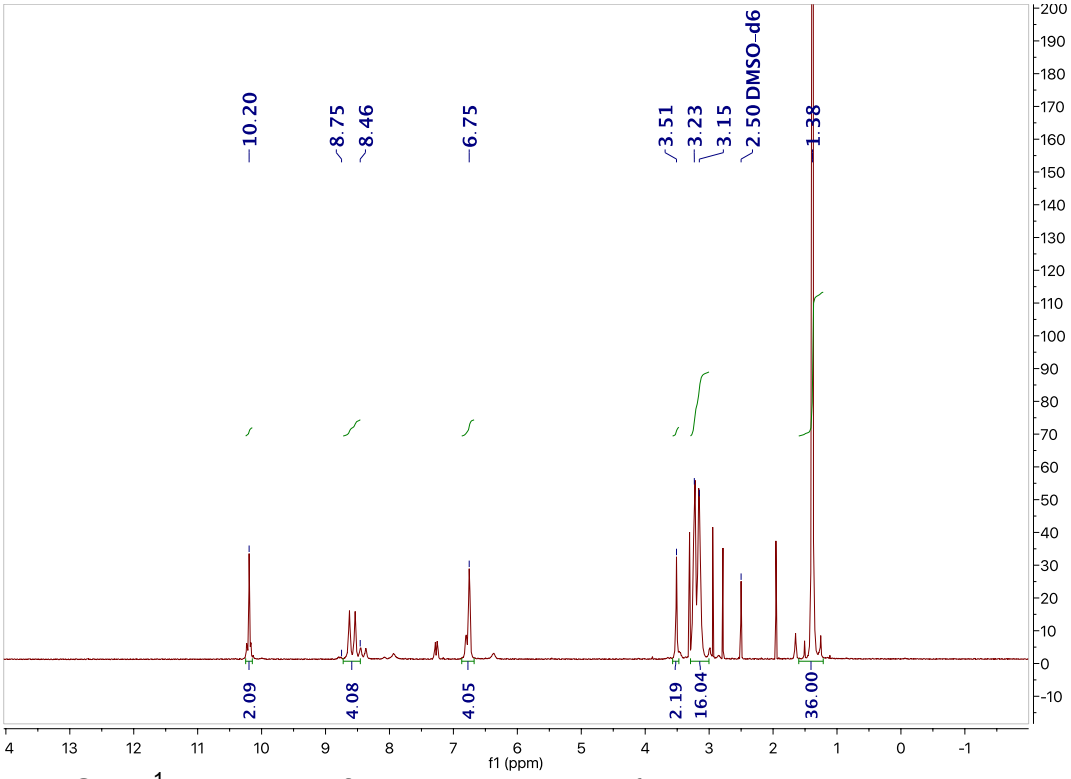

Figure S4. ^{1}H NMR of tetra-*tert*-butyl [({5,5'-[malonylbis(azanediyl)]bis(2,4,6-triiodoisophthaloyl)}tetrakis(azanediyl))tetrakis(ethane-2,1-diyl)]tetracarbamate **4**

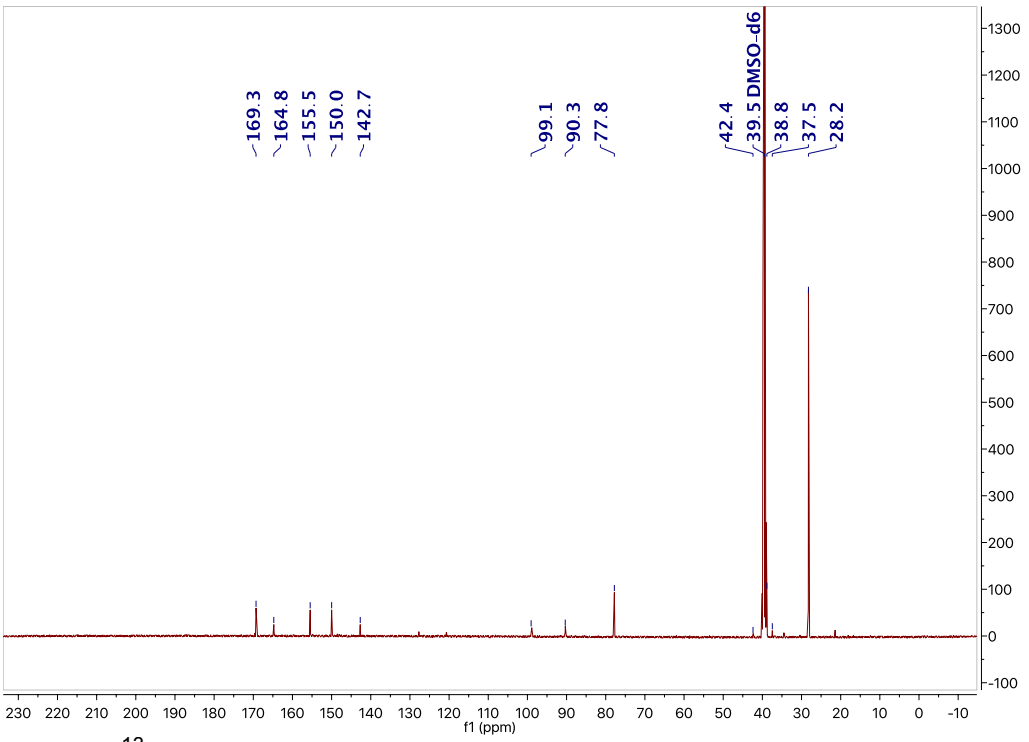

Figure S5. ^{13}C NMR of tetra-*tert*-butyl [({5,5'-[malonylbis(azanediyl)]bis(2,4,6-triiodoisophthaloyl)}tetrakis(azanediyl))tetrakis(ethane-2,1-diyl)]tetracarbamate **4**

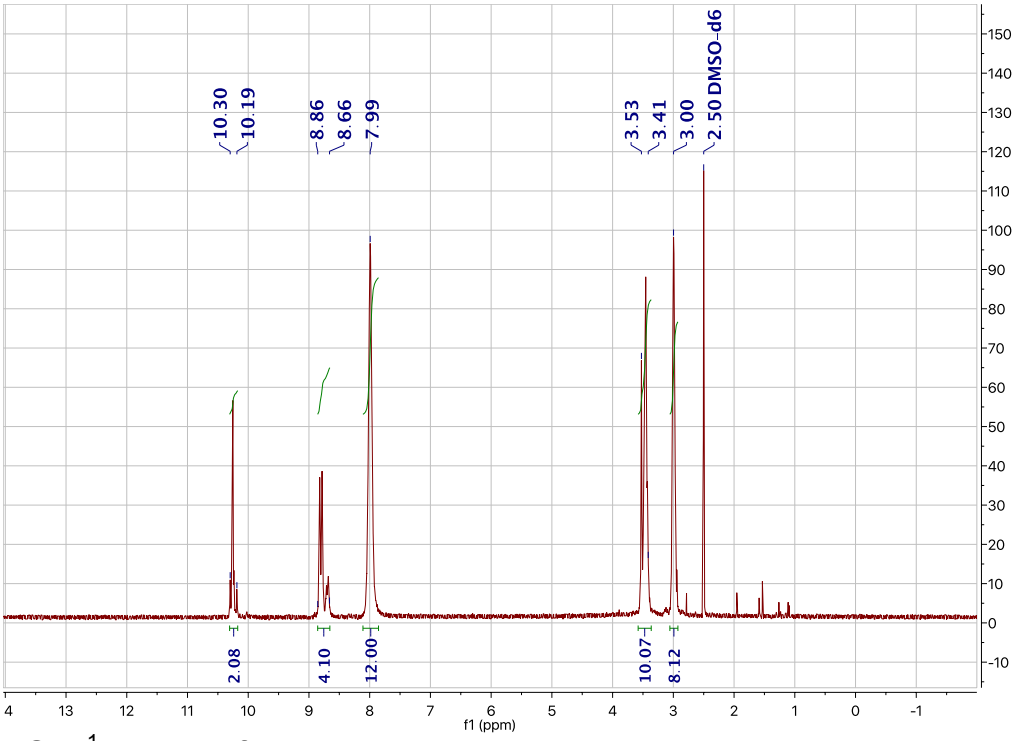

Figure S6. ^{1}H NMR of 5,5'-[malonylbis(azanediyl)]bis[*N1,N3*-bis(2-aminoethyl)-2,4,6-triiodoisophthalamide] TFA salt **5a**

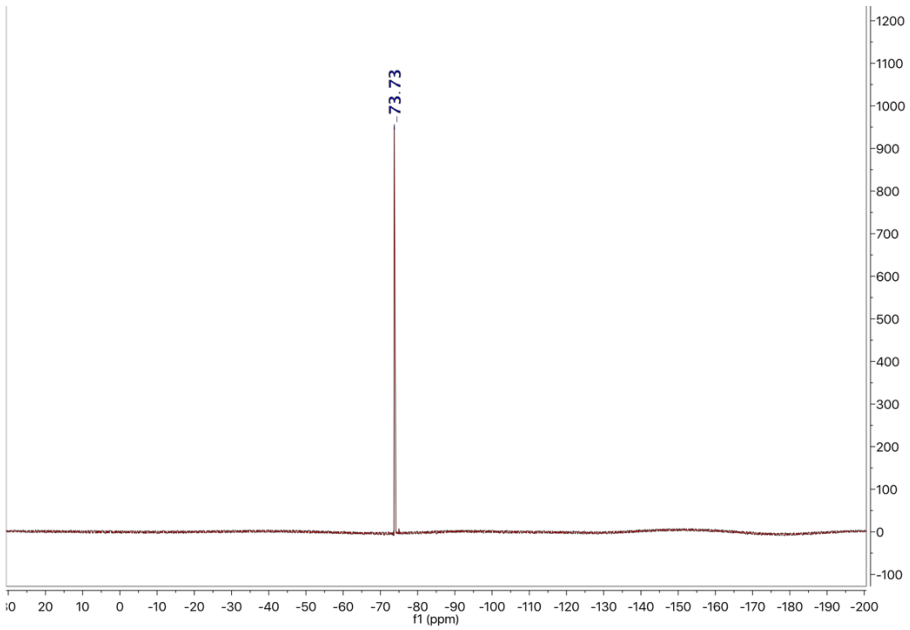

Figure S7. ^{19}F NMR spectra of 5,5'-[malonylbis(azanediyl)]bis[*N1,N3*-bis(2-aminoethyl)-2,4,6-triiodoisophthalamide] TFA salt **5a**

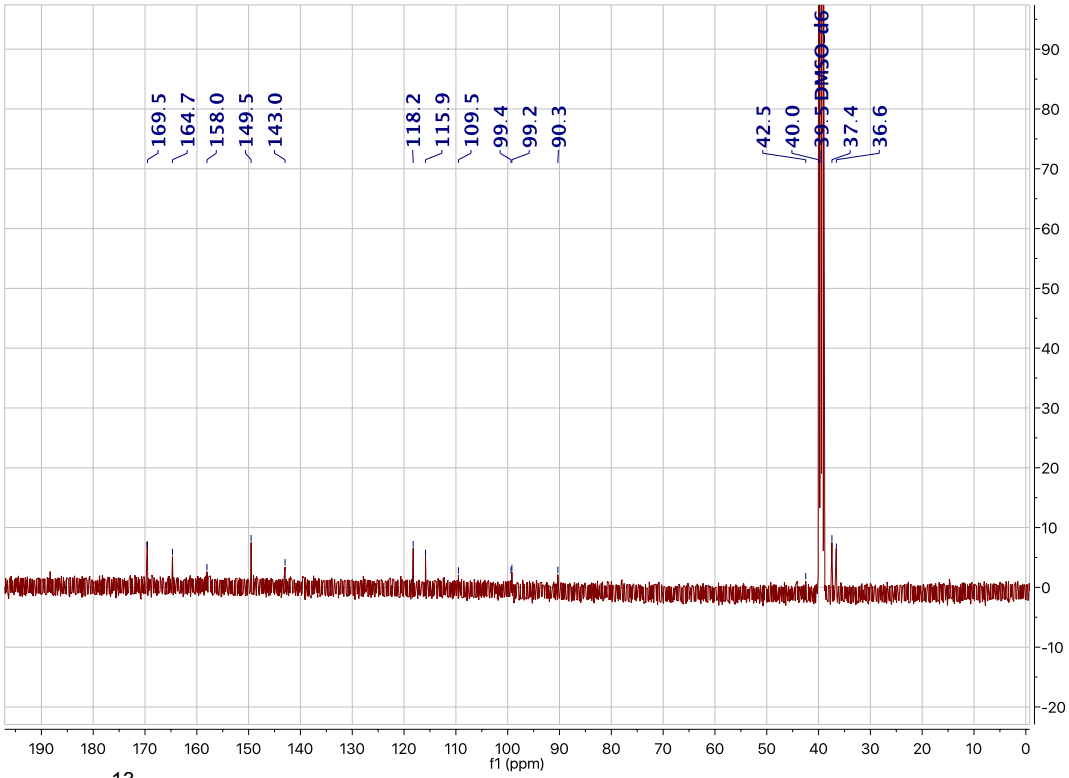

Figure S8. ^{13}C NMR of 5,5'-[malonylbis(azanediyl)]bis[*N1,N3*-bis(2-aminoethyl)-2,4,6-triiodoisophthalamide] TFA salt **5a**

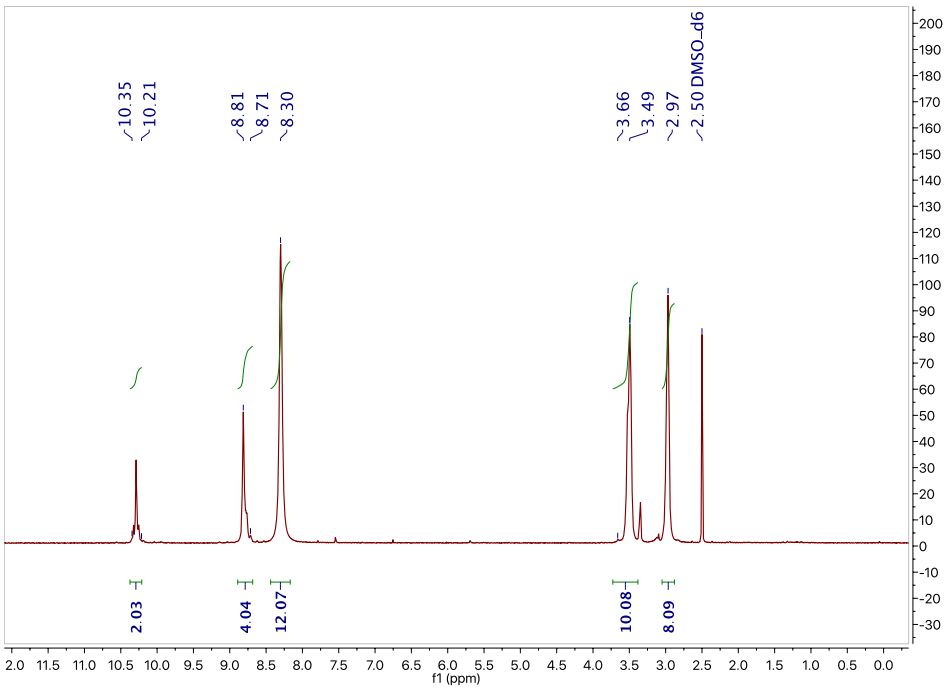

Figure S9. ^{1}H NMR of 5,5'-[malonylbis(azanediyl)]bis[*N1,N3*-bis(2-aminoethyl)-2,4,6-triiodoisophthalamide] chloride salt **5**

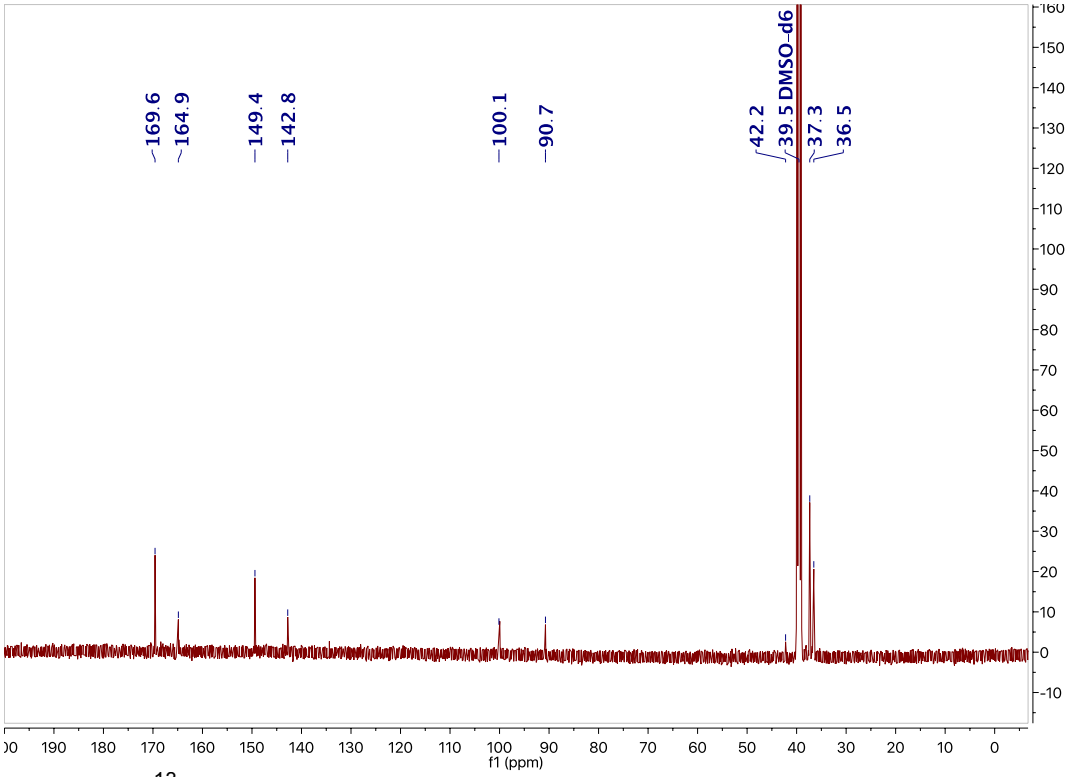

Figure S10. ^{13}C NMR of 5,5'-[malonylbis(azanediyl)]bis[*N1,N3*-bis(2-aminoethyl)-2,4,6-triiodoisophthalamide] chloride salt **5**

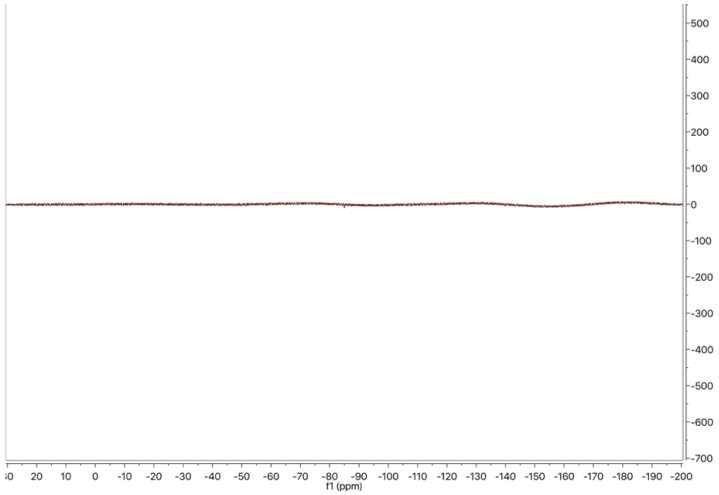

Figure S11. ^{19}F NMR spectra of 5,5'-[malonylbis(azanediyl)]bis[*N1,N3*-bis(2-aminoethyl)-2,4,6-triiodoisophthalamide] chloride salt **5**

HPLC analysis of 5,5'-[malonylbis(azanediyl)]bis[*N1,N3*-bis(2-aminoethyl)-2,4,6-triiodoisophthalamide] chloride salt **5 (**CA4+**)**

The purity of **5** salt was determined by analytical reverse-phase HPLC. Briefly, a Varian ProStar HPLC pump and UV-vis detector with Hamilton C18 HxSil 5mm 250x4.6 mm column was used with 1 mL/min flow rate of 95/5 water/ACN (isocratic). Product **5** was detected as a single peak at 245 nm with a retention time of 2.5 minutes. Shelf life of CA4+ **5** powder and aqueous solution of CA4+ **5** (12 or 24 mgI/mL, pH=7.4, 400 mOsm/kg), was determined by recording HPLC profile after one year. Both, CA4+ **5** powder and its aqueous solution remain unaltered (eluted as single peak at the same retention time) when stored for one year in refrigerator (+4 ºC). CA4+ **5** powder can be stored unaltered even at room temperature for a year.

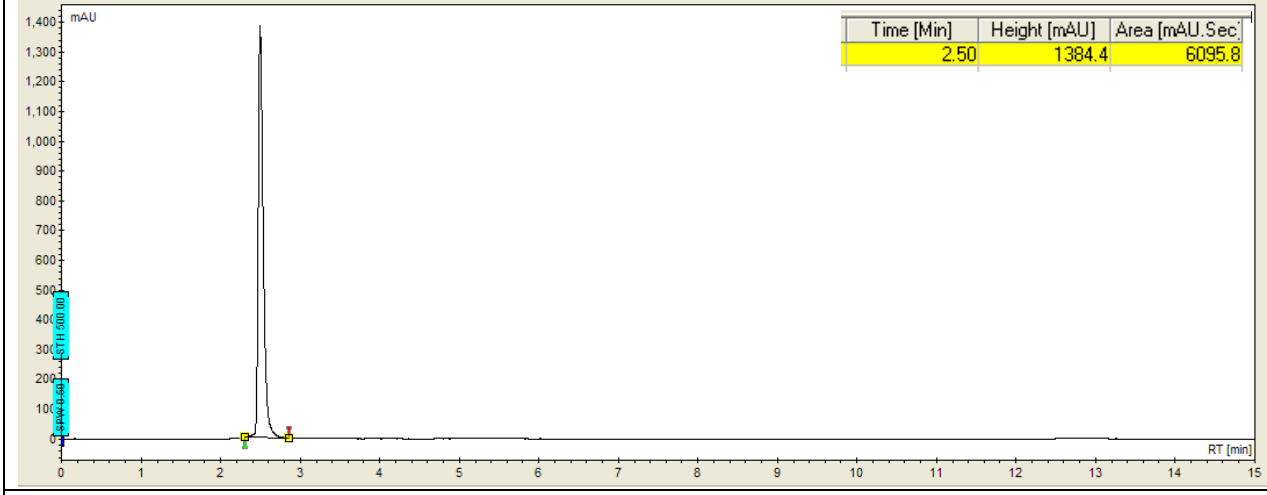

Figure S12. HPLC profile of 5,5'-[malonylbis(azanediyl)]bis[*N1,N3*-bis(2-aminoethyl)-2,4,6-triiodoisophthalamide] chloride salt **5**.

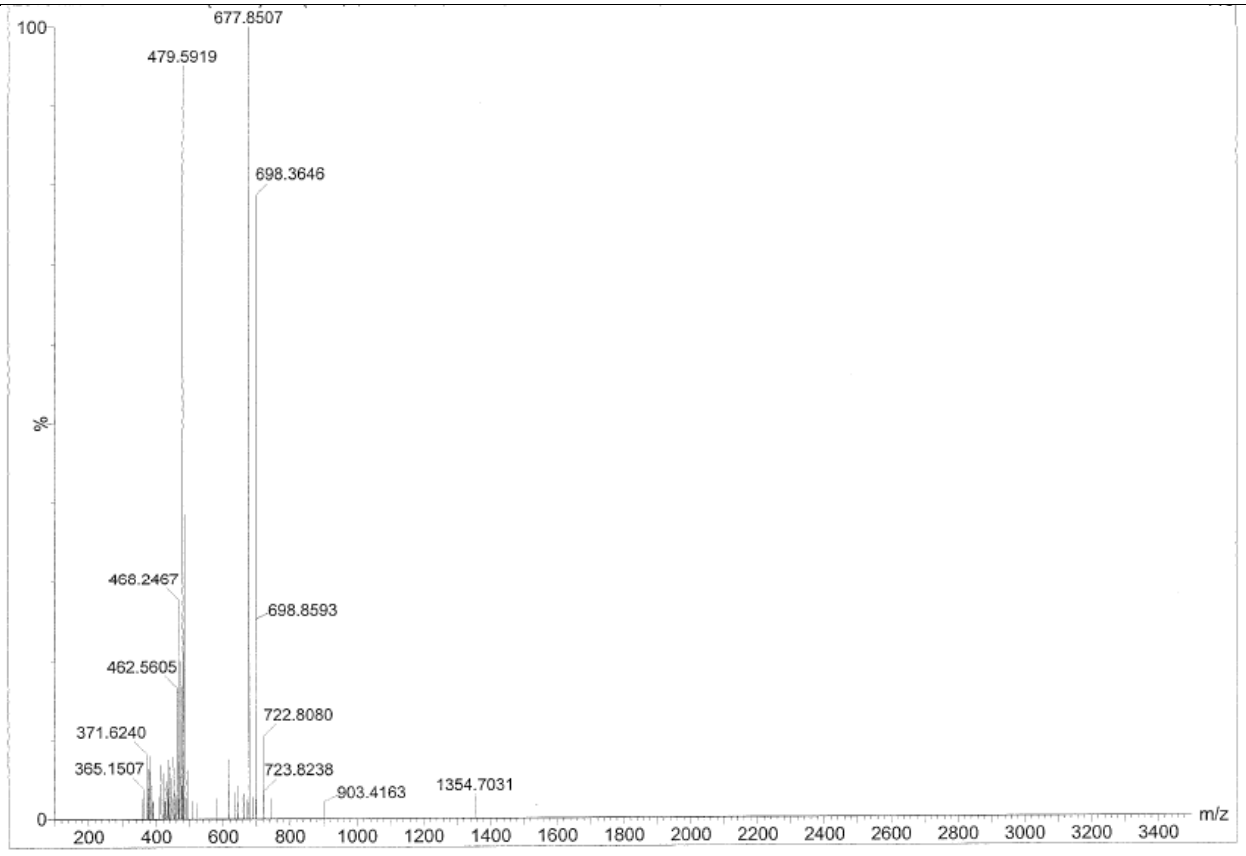

Figure S13. HRMS spectrum of 5,5'-[malonylbis(azanediyl)]bis[*N1,N3*-bis(2-aminoethyl)-2,4,6-triiodoisophthalamide] chloride salt **5**.

Table S1. Procedural differences between the previous synthesis of CA4+ (ref 45) and the current large scale synthesis of CA4+

	Old procedure (SI of Reference 46)	New procedure (This manuscript)
Step-1 Synthesis of **2**	❑ 3 g scale ❑ 30 equiv. thionyl chloride used ❑ removal of thionyl chloride on rotary evaporator (handling is difficult on large scale) ❑ excess use of ethyl acetate for extraction (50 mL/g of starting material **1**)	❑ 150 g scale ❑ 19.1 equiv. thionyl chloride used ❑ quenching of thionyl chloride (easy to handle) ❑ Use of ethyl acetate is almost one third (15 mL/g of starting material **1**) of previous usage ❑ Work-up is more convenient
Step-2 Synthesis of **3**	❑ Use of excess of hexanes for precipitation ❑ Yield was 60-65 % ❑ Pale yellow solid	❑ Precipitation was achieved by cooling at -20 °C ❑ Yield 89 % ❑ White solid (better purity than previous procedure)
Step-3 Synthesis of **4**	❑ Excess use of DMA (6 mL/g of **3**) ❑ Removal of DMA on rotary evaporator is very tedious (because of high boiling point of DMA) ❑ Use of excess of hexanes for precipitation ❑ Yield 61 %	❑ Use of DMA is half (3 mL/g of **3**) of previous usage ❑ No removal of DMA, precipitation in 1 mol/L aqueous HCl ❑ Removal of all excess reagent and bases through filtration ❑ Yield 89 %
Step-4 Synthesis of **5a**	❑ Use of excess of DCM:TFA (1:1) mixture ❑ Removal of DCM:TFA on rotary evaporator ❑ Product **5a** was obtained as glassy solid	❑ Usage of DCM:TFA (1:1) only 3.5 mL/g of **4** ❑ No use of rotary evaporator, product **5a** was precipitated out from ether ❑ Product **5a** was obtained as white free flow solid (easy to handle)
Step-5 Synthesis of **5**	❑ Lyophilization method was used ❑ Procedure was not optimized; yield was not measured	❑ Lyophilization was not convenient on larger scale, CA4+ **5** was precipitated from Acetone ❑ Optimization of the procedure and yield is more than 93 % (isolated)

Diffusion of Contrast Agents

Cationic, anionic and neutral contrast agents were diffused into six osteochondral plugs over 48 hours. Each contrast agent diffusion series was fit to the model:

$CECT\ Attenuation = \alpha(1 - e^{-\frac{t}{\tau}})$ (**Equation 1**).

Table S2. Equilibrium Attenuation and Time Constants as Fit to **Equation 1**.

Contrast Agent	Maximum attenuation (α) (HU)	Equilibrium attenuation (0.95α) (HU)	Time constant (τ) (hours)	Time required to reach equilibrium (t = 3τ) (hours)
CA4	2417	2291.15	12.5	37.5
Iodixanol	2053	1950.35	3.91	11.73
Ioxaglate	1918	1822.1	3.03	9.09

1,9-dimethylmethylene blue (DMMB) assay for glycosaminoglycan (GAG) content

A buffer solution of 50 mM sodium phosphate, 5 mM EDTA, 2 mM dithiothreitol (DTT), pH 6.8 was prepared for all solutions in this section. The cartilage was excised from the subchondral bone using a scalpel (#22 blade). Care was taken to remove all of the cartilage tissue by gently brushing the scalpel against the subchondral bone. The wet weight of the cartilage was measured, followed by lyophilization for 12 hours and then the dry weight of the cartilage was measured. Dry samples were digested in papain (0.5 mg/mL in the buffer solution) at 65 °C for 24 h. The GAG content of each cartilage sample was determined using the 1,9-dimethylmethylene blue (DMMB) colorimetric assay. A linear calibration curve (absorbance vs. GAG) was generated using concentrations of chondroitin-4-sulfate (Sigma 27042, St. Louis, MO) ranging from 10 to 100 mg/mL in the buffer solution. Each cartilage digestion solution was diluted 40 to 60 times for the assay. In triplicate, ten microliters of each chondroitin-4-sulfate calibration solution and each diluted sample digestion solution were added and mixed with 100 µL of DMMB dye solution in a 96-well plate. The absorbance (520 nm) was measured using a plate reader (Beckman Coulter AD340, Fullerton, CA). The total GAG mass of each sample was calculated using the calibration curve and a GAG concentration was computed by dividing the total GAG mass (mg) by the wet weight (mg) of the cartilage.

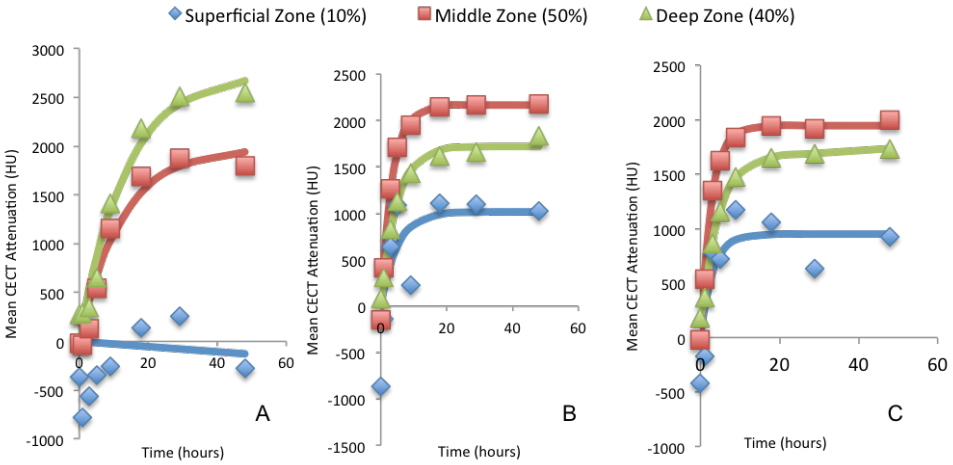

Figure S14. Diffusion and partition of Ioxaglate, Iodixanol and CA4+ into bovine osteochondral plugs divided into superficial, middle and deep zones of cartilage.

Contrast agents with different valance charges were diffused into bovine osteochondral plugs (n = 6) over 48 hours: **(A)** CA4+ with +4 valance, **(B)** Iodixanol, a neutral agent, and **(C)** Ioxaglate with a -1 valance. The cartilage was segmented into three different zones: superficial zone (top 10% from articular surface), middle zone (middle 50%) and deep (40% from bone-cartilage interface). Both Iodixanol and Ioxaglate appear to diffuse throughout the cartilage tissue with the greatest contribution from the middle zone and secondarily from the deep zone of cartilage with a lesser contribution from the superficial zone. For CA4+, the main zone of highest attenuation is the deep zone followed by the middle zone, with no significant contribution from the superficial zone.

The attenuation and equilibrium time calculated from **Equation 1** is summarized for each cartilage zone in **Table S2**.

Table S3. Equilibrium Attenuation and Time Constants as Fit to **Equation 1** by Zone of Cartilage.

Contrast Agent		Maximum attenuation (α) (HU)	Equilibrium attenuation (0.95α) (HU)	Time constant (τ) (hours)	Time required to reach equilibrium ($t = 3\tau$) (hours)
CA4+	Superficial Zone (10%)	-40350	-38332.50	15200	45510
	Middle Zone (50%)	1979	1880.05	12.21	36.63
	Deep Zone (40%)	2745	2607.75	13.37	40.11
Iodixanol	Superficial Zone (10%)	1017	966.15	4.97	14.92
	Middle Zone (50%)	2167	2085.65	3.54	10.61
	Deep Zone (40%)	1721	1634.95	4.79	14.36
Ioxaglate	Superficial Zone (10%)	950	902.60	3.07	9.21
	Middle Zone (50%)	1946	1848.70	2.76	8.27
	Deep Zone (40%)	1700	1615.00	4.31	12.92

Table S4. Individual clinical chemistry parameters

	Sex	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)	Calcium 2 (mmol/L)	TG (mg/dL)	CHOL (mg/dL)	TP (g/dL)	ALB (g/dL)	GLOB (g/dL)	A/G	TB (mg/dL)
Group 1	Male	143	10.59	98.4	12.2	44	99	7.0	4.4	3	2	0.1
	Male	141	12.00	96.5	13.0	30	97	7.4	4.8	3.0	2	0.1
	Male	140	11.08	95.1	14.0	60	108	8.1	5.2	3.0	2	0.1
	Female	139	14.23	98.7	11.9	48	94	7.1	4.7	2.0	2	0.1
	Female	139	13.13	97.2	12.3	54	109	7.1	4.9	2.0	2	0.1
	Female	141	11.74	95.4	12.6	59	151	7.7	4.9	3.0	2	0.1
Group 2	Male	141	11.20	97.0	12.6	42	114	7.5	4.9	3.0	2	0.1
	Male	140	10.94	94.6	13.7	81	156	7.6	4.8	3.0	2	0.1
	Male	143	9.88	93.6	14.4	64	129	8.5	5.5	3.0	2	0.1
	Female	136	16.32	97.6	12.7	57	95	7.1	5.0	2.0	2	0.1
	Female	138	14.54	98.2	12.8	48	105	7.5	5.0	3.0	2	0.1
	Female	143	10.88	97.8	13.7	48	141	8.0	5.4	3.0	2	0.1
Group 3	Male	139	9.98	91.5	15.1	64	131	9.4	5.9	4.0	2	0.1
	Male	144	9.38	92.4	14.6	88	156	8.6	5.3	3.0	2	0.1
	Male	139	12.82	95.7	13.8	63	115	8.2	5.2	3.0	2	0.1
	Female	138	13.66	98.9	12.6	50	145	7.6	5.4	2.0	2	0.1
	Female	139	12.87	97.9	12.3	45	125	7.2	4.9	2.0	2	0.1
	Female	139	11.96	96.9	12.9	60	146	7.6	5.2	2.0	2	0.1
Group 4	Male	139	12.96	94.8	13.0	69	120	7.4	4.6	3.0	2	0.1
	Male	141	11.44	95.9	14.2	58	129	7.8	4.8	3.0	2	0.1
	Male	144	10.13	93.4	16.6	84	152	10.1	6.8	4.0	2	0.1
	Female	137	12.25	97.7	12.5	70	121	6.8	4.7	2.0	2	0.1
	Female	140	10.81	96.7	13.8	73	133	8.4	5.6	3.0	2	0.1
	Female	142	11.03	97.2	13.3	58	166	8.4	5.6	3	2	0.1

Na = Sodium
K = Potassium
Cl = Chloride
TG = Triglycerides
TB = Total Bilirubin
ALB = Albumin
TP = Total Protein
Ca = Calcium
GLOB = Globulin (= TP- ALB)
A/G = Alb/Glob ratio
CHOL = Cholesterol

Table S5. Individual clinical chemistry parameters

	Sex	PHOS (mg/dL)	GLU (mg/dL)	BUN (mg/dL)	CREAT (mg/dL)	ALK (U/L)	ALT (U/L)	AST (U/L)	GGT (U/L)	Lipemia	Hemolysis	Icteric
Group 1	Male	14.8	154	21	0.41	105	58	93	-3	11	32	0
	Male	16.3	145	19	0.36	108	82	126	-2	6	25	0
	Male	15.1	199	16	0.38	159	81	136	-2	9	24	0
	Female	12.5	90	20	0.45	56	68	131	-3	5	54	0
	Female	12.6	92	17	0.41	53	56	83	-2	7	24	0
	Female	12.8	83	18	0.43	73	62	102	-2	11	27	0
Group 2	Male	13.2	223	23	0.38	119	93	97	-3	10	27	0
	Male	12.4	282	18	0.36	145	67	85	-1	8	18	0
	Male	14.3	258	18	0.40	140	63	81	-1	4	16	0
	Female	15.4	75	19	0.47	79	73	114	-2	7	24	0
	Female	13.8	153	19	0.44	61	59	92	-4	7	85	0
	Female	14.2	87	19	0.42	81	53	101	-1	10	20	0
Group 3	Male	13.4	306	23	0.43	148	82	105	-2	8	21	0
	Male	14.1	274	17	0.36	117	59	93	-1	9	16	0
	Male	14.2	156	20	0.35	145	93	125	-2	8	26	0
	Female	11.0	119	17	0.43	87	42	90	-2	10	22	0
	Female	12.3	87	18	0.39	70	68	91	-1	8	21	0
	Female	12.0	103	18	0.38	67	63	81	-2	13	18	0
Group 4	Male	15.9	176	21	0.41	113	324	533	-3	6	101	0
	Male	15.9	219	18	0.39	157	60	94	-4	8	61	0
	Male	16.1	273	20	0.44	193	87	101	-5	9	52	0
	Female	12.1	138	19	0.38	65	59	99	-4	10	66	0
	Female	12.7	138	18	0.47	93	64	103	-2	8	30	0
	Female	10.9	117	16	0.46	101	66	95	-3	14	37	0

ALK = Alkaline Phosphatase
ALT = Alanine Aminotransferase
AST = Aspartate Aminotransferase
PHOS = Phosphorus (inorganic)
GLU = Glucose
GGTL = Gamma-Glutamyl Transferase Level
CREAT = Creatinine
BUN = Blood Urea Nitrogen

Table S6. Normal values, Sprague Dawley 15-16 week old males (N = 20)

Clinical Chemistry

	Units	Mean	Std Dev
Glucose	mg/dL	118.4	30.4
Blood Urea Nitrogen (BUN)	mg/dL	17.8	1.9
Creatinine	mg/dL	0.4	0.1
BUN/Creatinine	Ratio	45.6	6.6
Cholesterol	mg/dL	114.9	11.6
Total Protein (TP)	g/dL	6.5	0.2
Albumin (Alb)	g/dL	4.2	0.2
Globulin (Glb)	g/dL	2.3	0.1
Alb/Glb	Ratio	1.8	0.1
Total Bilirubin	mg/dL	0.1	0.0
Alkaline Phosphatase (ALP)	U/L	65.5	10.1
Aspartate Aminotransferase (AST)	U/L	118.1	56.7
Alanine Aminotransferase (ALT)	U/L	56.7	11
AST/ALT	Ratio	2.1	0.9
Calcium (Ca)	mg/dL	10.9	0.5
Phosphorus (P)	mg/dL	9.0	0.7
Sodium (Na)	mmol/L	146.8	2.9
Potassium (K)	mmol/L	6.4	0.7
Chloride (Cl)	mmol/L	96.7	2.4
HCO_3	mmol/L	42.4	3.6
Anion Gap	mmol/L	14.2	3.9

Hematology

	Units	Mean	Std Dev
White Blood Cells (WBC)	x 10^3/uL	7.5	2.6
Red Blood Cells (RBC)	x 10^6/uL	9.3	0.5
Hemoglobin (Hgb)	g/dL	17.2	0.9
Hematocrit (Hct)	%	58.1	2.8
Mean Corpuscular Volume (MCV)	fL	62.3	1.3
Mean Corpuscular Hemoglobin (MCH)	pg	18.4	0.5
Mean Corpuscular Hemoglobin Concentration (MCHC)	g/dL	29.6	0.4
Red Cell Distribution	%	12.4	0.5
Mean Platelet Volume (MPV)	fL	0.9	0.6
Segmented Neutrophils	%	12.1	5.2

Table S7. Clinical Chemistry parameters (normal values, Sprague Dawley 15-16 week old females (N = 20))

Clinical Chemistry

	Units	Mean	Std Dev
Glucose	mg/dL	92.2	16.6
Blood Urea Nitrogen (BUN)	mg/dL	17.1	2.8
Creatinine	mg/dL	0.4	0.1
BUN/Creatinine	Ratio	39.7	3.8
Cholesterol	mg/dL	102.8	21.1
Total Protein (TP)	g/dL	6.4	0.3
Albumin (Alb)	g/dL	4.4	0.2
Globulin (Glb)	g/dL	2.0	0.2
Alb/Glb	Ratio	2.2	0.2
Total Bilirubin	mg/dL	0.2	0.1
Alkaline Phosphatase (ALP)	U/L	52.5	20
Aspartate Aminotransferase (AST)	U/L	172	110.1
Alanine Aminotransferase (ALT)	U/L	49.7	11.3
AST/ALT	Ratio	3.3	1.5
Calcium (Ca)	mg/dL	10.7	0.7
Phosphorus (P)	mg/dL	8.8	0.9
Sodium (Na)	mmol/L	144.1	2.7
Potassium (K)	mmol/L	6.7	1.0
Chloride (Cl)	mmol/L	97.6	2.1
HCO_3	mmol/L	38.9	4.0
Anion Gap	mmol/L	14.4	4.1

Hematology

	Units	Mean	Std Dev
White Blood Cells (WBC)	x 10^3/uL	6.7	2.0
Red Blood Cells (RBC)	x 10^6/uL	8.6	0.4
Hemoglobin (Hgb)	g/dL	16.5	0.8
Hematocrit (Hct)	%	54.2	2.6
Mean Corpuscular Volume (MCV)	fL	63.2	1.5
Mean Corpuscular Hemoglobin (MCH)	pg	19.2	0.5
Mean Corpuscular Hemoglobin Concentration (MCHC)	g/dL	30.4	0.4
Red Cell Distribution	%	12.1	0.7
Segmented Neutrophils	%	10.3	4.0
Lymphocytes	%	86.2	4.5
Monocytes	%	1.8	0.9
Eosinophils	%	1.3	0.9

Technical data sheet for ^{14}C-labeled CA4+ **5***.

Specific Activity: 8.7 mCi/mmol (5.82 μCi/mg)
by gravimetric analysis
Identification by MS: Conforms

Packaged: 151.3 μCi (26 mg), white solid

Total Batch Activity: 151.3 μCi
Reserve: 0.0 μCi

Radiochemical Purity: >99% by HPLC on 03/12/2014
Chemical Purity: >98% by HPLC (UV_{245}) on 03/12/2014

Stability and Storage Recommendation: The exact rate of decomposition is unknown. However, it can be assumed that the product may decompose at a rate of approximately 1% per month when stored at -20° C in the original container.

HPLC
Luna C18, 5μm, 250 x 4.6mm
Mobile Phase: 0.05% TFA in water
Flow Rate: 1.0 mL / min., RC Flow Det.
Isocratic

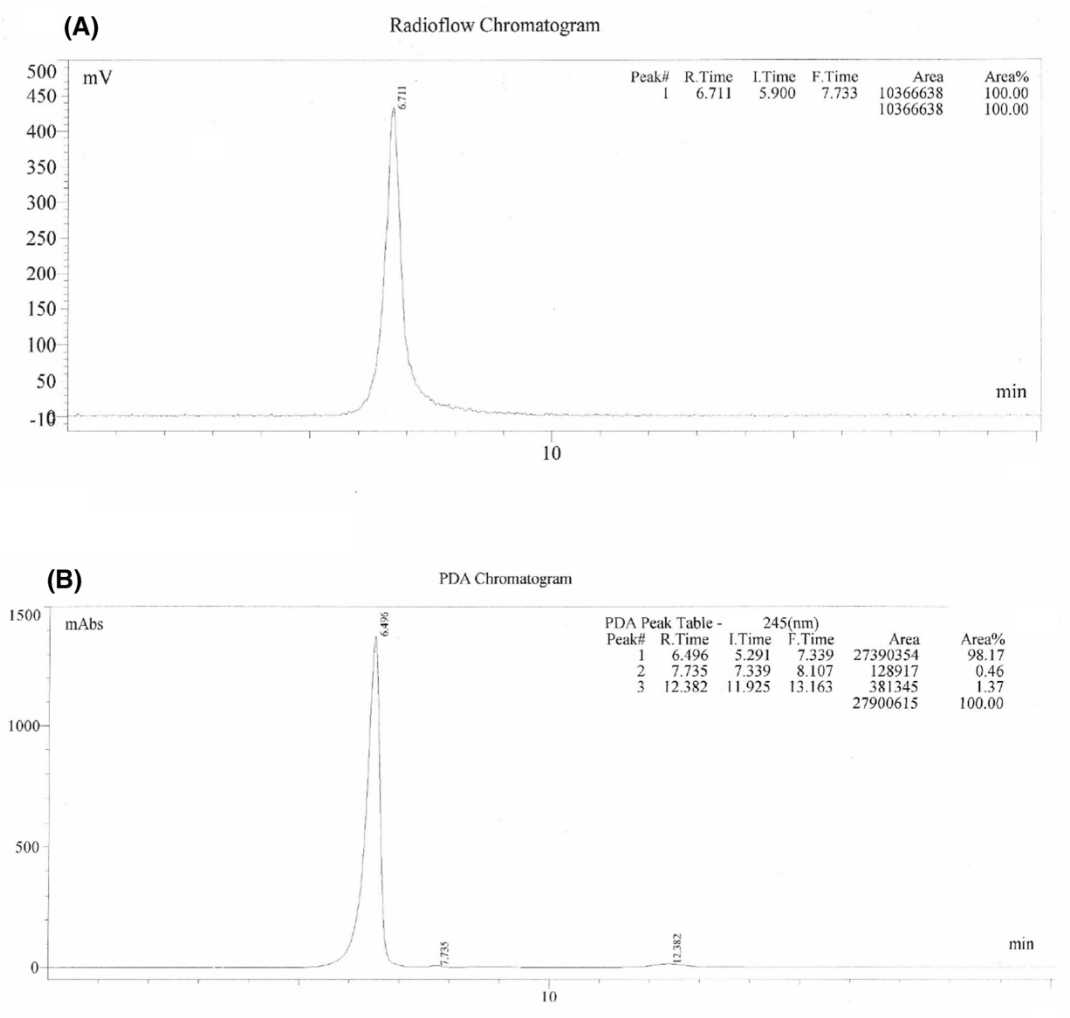

Figure S15. (A) Radioflow chromatogram (for radiochemical purity) and (B) PDA chromatogram (for chemical purity) for ^{14}C-labeled CA4+ **5***