Supplementary Information

Azide- and Dye-Conjugated Coelenterazine Analogues for a Multiplex Molecular Imaging Platform

Ryo Nishihara,[1] Emi Hoshino,[1] Yoshiki Kakudate,[1] Satoshi Kishigami,[1] Naoko Iwasawa,[1] Shin-ichi Sasaki,[2] Takahiro Nakajima,[3] Moritoshi Sato,[3] Shigeru Nishiyama,[1] Daniel Citterio,[1] Koji Suzuki*,[1] Sung Bae Kim*,[4]

1. Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8522, Japan
2. Nagahama Institute of Bio-Science and Technology, Nagahama, Kusatsu, Shiga 525-8577, Japan
3. Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan
4. Research Institute for Environmental Management Technology, National Institute of Advanced Industrial Science and Technology (AIST), 16-1 Onogawa, Tsukuba 305-8569, Japan

*** Corresponding authors:**

suzuki@applc.keio.ac.jp

kimu-sb@aist.go.jp

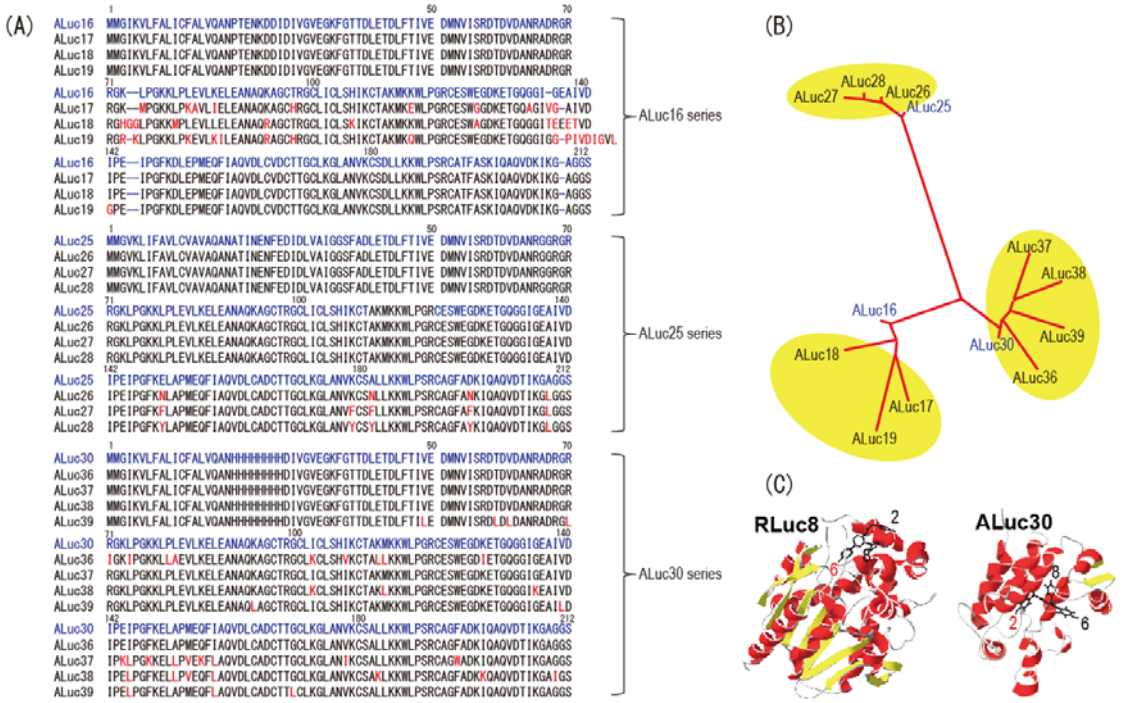


Figure S1. (A) The sequences of ALucs, newly synthesized in this study. The sequences in blue and black indicate, respectively, the prototypes and the sibling sequences. Letters in red highlight the amino acids that are different from others. (B) The phylogenetic tree of newly fabricated ALucs, derived from ALuc25, ALuc16, and ALuc30. The original ALucs were marked in blue. (C) The molecular structures of two marine luciferases, *Renilla reniformis* luciferase 8 (RLuc8) (PDB: 2PSJ) and artificial luciferase 30 (ALuc30; GenBank MF817970) (*11*) and their binding with coelenteramide and native coelenterazine (nCTZ), respectively. The molecular structure of ALuc30 is a supersecondary model, which was calculated with respect to the X-ray crystallographic information of the coelenterazine-binding protein (CBP) (PDB**:** 2hps and 2hq8) (*11*)

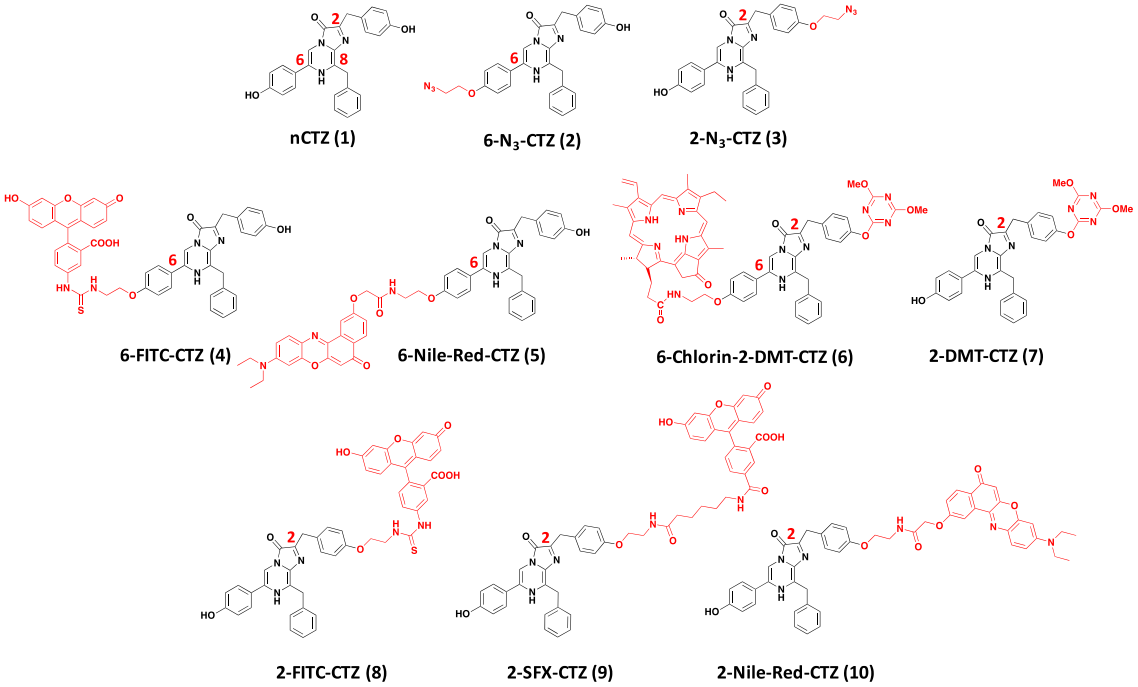


Figure S2. Chemical structures of novel coelenterazine analogues, synthesized for marine luciferases in the present study. The CTZ analogues were dye-bridged at C-2 or C-6-positions. Abbreviations: nCTZ, native coelenterazine; N_3, an azide group; FITC, fluorescein isothiocyanate; SFX, fluorescein succinimidyl ester; DMT, 4,6-dimethoxy-1,3,5-triazin-2-yl. The modified functional groups in the chemical structures are highlighted in red.

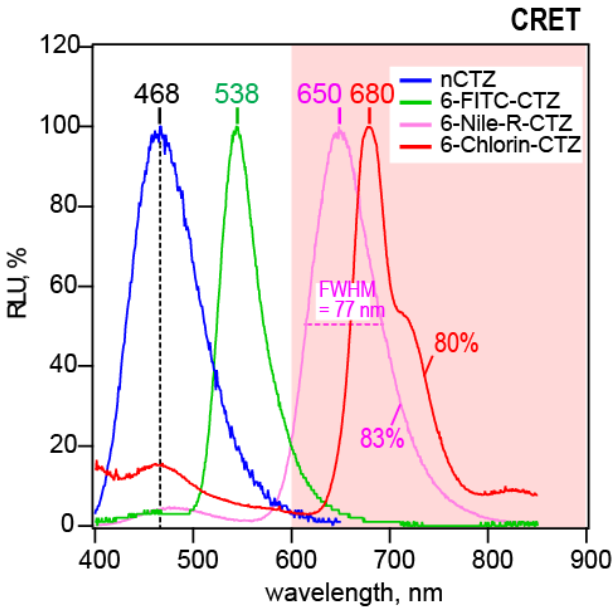


Figure S3. The chemiluminescence resonance energy transfer (CRET) spectra of nCTZ analogues dye-conjugated at the C-6 position. The spectra were normalized as percentages (%) of maximal intensity. The percentage in red denotes the portion of red light emission longer than 600 nm over the total light emission. Among tested, some spectra were omitted in the figure, because of the poor absolute optical intensities. The FWHM means the full width at half maximal intensity in wavelength (nm).

	0.1 mM																1 mM			
	nCTZ		2-N3-CTZ		2-FITC-CTZ		2-SFX-CTZ		2-Nile-R-CTZ		6-N3-CTZ		6-FITC-CTZ		2-DMT-CTZ		2-DMT-6-Chlorin-CTZ		6-Nile-R-CTZ		2-SFX-CTZ		6-Nile-R-CTZ	
	ave	SD	ave	SD	ave	SD	ave	SD	ave	SD	ave	SD	ave	SD	ave	SD	ave	SD	ave	SD	ave	SD	ave	SD
GLuc	102	± 36	7	± 1	2	± 0	2	± 1	0	± 1	1	± 0	1	± 0	1	± 1	0	± 1	2	± 1	3	± 0	1	± 1
RLuc8.6-535	71	± 5	3	± 1	3	± 0	2	± 1	0	± 1	15	± 3	96	± 20	2	± 1	1	± 1	1	± 1	3	± 0	2	± 1
ALuc16	529	± 128	876	± 67	4	± 4	3	± 1	1	± 1	2,097	± 326	74	± 5	2	± 1	1	± 1	0	± 2	3	± 0	2	± 0
ALuc22	797	± 91	723	± 52	4	± 1	3	± 0	1	± 0	1,612	± 87	96	± 16	2	± 0	2	± 1	2	± 0	4	± 0	2	± 1
ALuc23	711	± 230	1,718	± 49	6	± 3	2	± 1	1	± 0	3,092	± 612	154	± 37	2	± 1	1	± 1	1	± 1	3	± 0	2	± 1
ALuc24	498	± 166	359	± 30	0	± 1	3	± 1	1	± 1	690	± 188	35	± 2	2	± 0	1	± 1	3	± 0	3	± 1	2	± 1
ALuc30	351	± 91	716	± 68	2	± 2	2	± 1	0	± 1	1,019	± 36	84	± 5	2	± 0	1	± 1	2	± 0	3	± 0	2	± 0
ALuc34	1,218	± 386	2,256	± 277	10	± 4	2	± 1	0	± 1	4,205	± 246	249	± 39	1	± 1	1	± 1	38	± 16	3	± 0	2	± 1

RLU/sec/mm^2

Table S1. Absolute optical intensities (RLU/sec/mm^2) of the newly synthesized azide- or dye-conjugated CTZ analogues according to luciferases, shown in Figure 1(B) (*n*=3). Two different concentrations of the substrates were applied for the light measurement: i.e., 0.1 mM and 1 mM. Accordingly, the results were grouped into 0.1 and 1 mM areas. Light- and dark-colored spots show the optical intensities higher than 15 and 1,500 counts (RLU/sec/mm^2), respectively.

RLU/sec/mm^2

		nCTZ			6-pi-OH-CTZ			2-N3-CTZ			6-N3-CTZ		
	min	ave	SD	%	ave	SD	%	ave	SD	%	ave	SD	%
GLuc	0	8,979	2,459	32	35	6	17	608	127	8	172	33	16
	20	2,906	716		6	2		48	9		28	6	
RLuc8.6 −535	0	6,757	4,021	63	**10,113**	1,340	57	269	28	52	364	73	33
	20	4,245	2,569		5,813	829		139	12		119	27	
ALuc17	0	8,416	874	38	198	23	41	2,205	130	62	2,517	339	11
	20	3,227	425		81	11		1,360	97		285	32	
ALuc18	0	**55,593**	1,836	16	477	40	24	**9,623**	1,800	23	6,721	675	1
	20	8,957	436		117	8		2,230	446		46	3	
ALuc19	0	14,740	2,968	41	5,838	1,134	29	13,245	772	54	22,893	3,429	8
	20	5,997	1,098		1,710	280		7,136	443		1,732	222	
ALuc26	0	**53,263**	7,413	**64**	**14,399**	2,284	**52**	**23,002**	5,325	**47**	**57,578**	1,602	**27**
	20	33,844	9,100		7,458	997		10,792	2,256		15,572	2,058	
ALuc27	0	68	28	31	22	10	36	28	16	21	94	38	15
	20	21	10		8	4		6	4		14	6	
ALuc28	0	496	67	59	43	15	49	31	6	65	265	35	15
	20	292	47		21	8		20	4		41	4	
ALuc36	0	262	19	93	4	1	75	47	7	40	75	16	40
	20	243	26		3	1		19	3		30	10	
ALuc37	0	229	37	61	0	0	0	2	1	50	11	3	55
	20	140	35		0	0		1	1		6	2	
ALuc38	0	199	33	1	0	1	0	1	3	1	14	3	1
	20	2	52		0	1		0	1		0	2	
ALuc39	0	2,788	964	46	949	161	23	4,237	661	43	4,175	236	21
	20	1,287	473		216	33		1,841	269		861	72	

Table S2. Absolute optical intensities (RLU/sec/mm^2) of the newly synthesized azide-conjugated CTZ analogues according to new ALucs, shown in Figure 2(B) (n=4). The percentage (%) indicates the optical intensity that remained at 20 minutes after substrate injection, compared to what emitted at 0 minute after substrate injection. The bold numbers highlight dominant optical intensities of luciferases or luciferins. The dotted line in red marks the zone of poor optical intensity.

Supplementary Experimental Procedures

Experimental Procedure S1

The sequences of new ALuc variants were made by our previously suggested method (*1*) (*2*). Briefly, the sequence of ALuc25 or ALuc30 was fragmented and aligned to make three lanes with CLUSTALW ver2.1 to investigate their internal sequential homology (named Single-Sequence Alignment (SSA)) (*1*). This alignment specified characteristic three repeated lanes, whose second and third lanes were highly conserved. The new ALuc sequences were generated by replacing the original amino acids in the alignment with new candidates to enhance the homology between the second- and third-lane sequences. The new ALuc sequences derived from ALuc25 and ALuc30 were named ALuc26–28, and ALuc36–39, respectively (Figure S1). The corresponding GenBank accession numbers are as follows: ALuc26, MF958968; ALuc36, MF958970; ALuc39, MF958971.

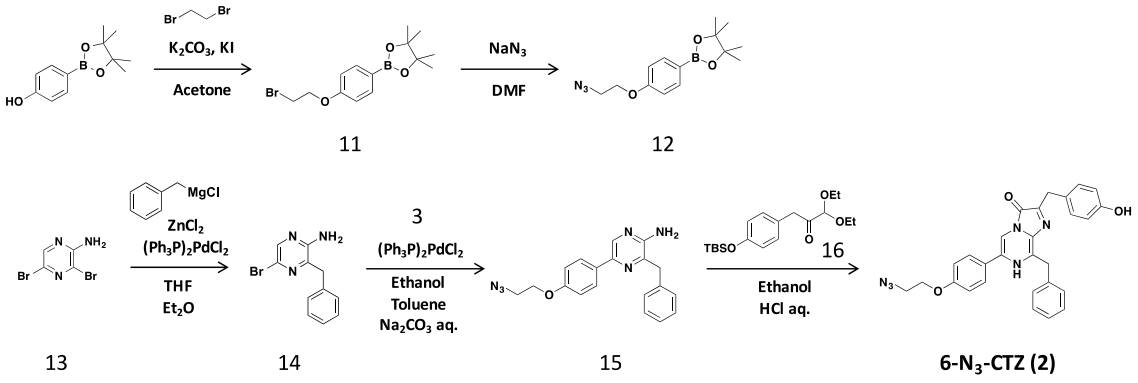
Based on the sequential information above, the murine codon-optimized cDNA constructs, encoding the artificially designed amino acid sequences (ALuc26–28 and ALuc36–39), were custom-synthesized, on order, by Eurofins Genomics (Tokyo, Japan). The synthesized cDNAs encoding each luciferase were subcloned into pcDNA3.1(+)

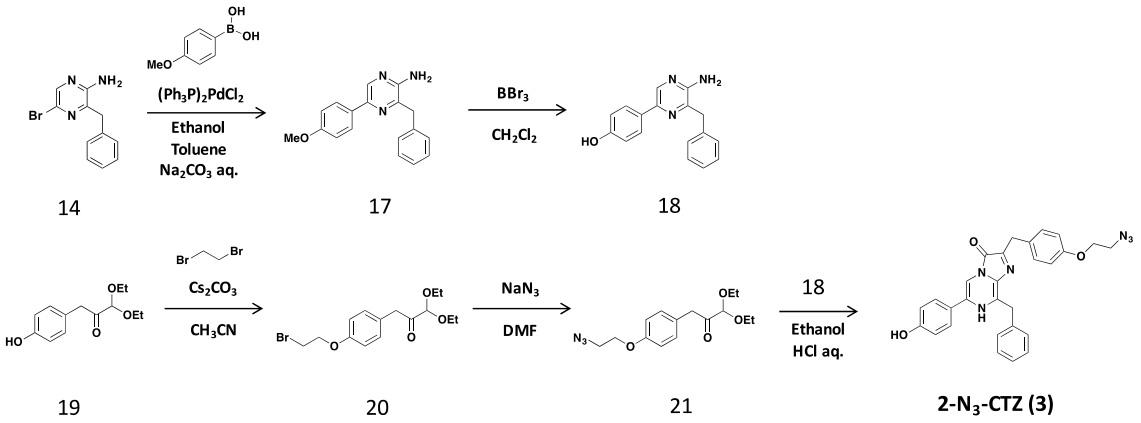
(Invitrogen), using the specific restriction sites, *Hind*III and *Xho*I, for expression in mammalian cells, where the KEDL was added to the end of the sequence of each luciferase for the cell retention. The overall sequence fidelity was confirmed with a sequencing service provided by Eurofins Genomics (Tokyo, Japan).

Experimental Procedure S2

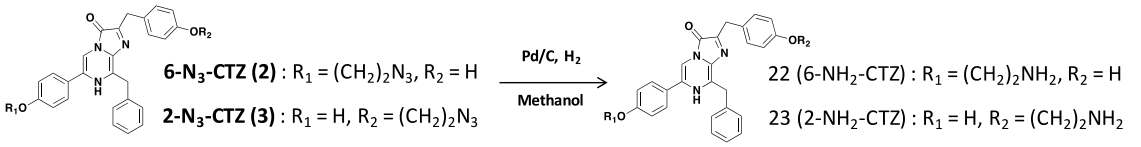
Fabrication of new ALuc variants for screening their best matches with azide-conjugated CTZ analogues

The C-2 and C-6 modified CTZ derivatives were newly synthesized according to the synthesis routes reported in literatures [3] [4]. The major synthetic schemes of selected CTZ analogues are as follows:



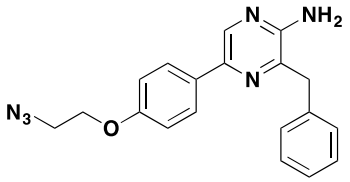


The synthesis scheme of 6-N_3-CTZ and 2-N_3-CTZ (Compounds 11-14 and 16-19 are reported previously (*3*)) Compounds 20-21 are synthesized in reference to previous work (*2*).



The synthesis scheme of NH_2-modified CTZ analogues (6-NH_2-CTZ and 2-NH_2-CTZ)

5-(4-(2-Azidoethoxy)phenyl)-3-benzylpyrazin-2-amine (15)

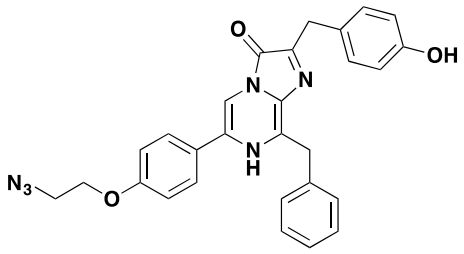


3-Benzyl-5-bromopyrazin-2-amine (**14**) (660 mg, 2.50 mmol, 1.0 eq.) and 2-(4-(2-azidoethoxy)phenyl) -4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**12**) (1.0 g, 3.46 mmol, 1.6 eq.) were dissolved in toluene (15 ml) and stirred at room temperature. Ethanol (5 ml)

and 1 M sodium carbonate aq. (7 ml) were added into the reaction mixture. After vacuum deaeration, a catalytic amount of tetrakis(triphenylphosphine)palladium(0) was added into the solution and the mixture was deaerated again and stirred for 20 hours at 100 ℃. After cooling to room temperature, the solution was filtered through a Celite pad to remove the palladium catalyst. The solution was extracted with ethyl acetate, and the brown organic phase was washed with water, sat. sodium hydrogen carbonate aq. and brine, dried over sodium sulfate and evaporated. The resulting residue was purified by means of silica gel column chromatography (eluent composition: chloroform/ethyl acetate = 19/1 to 9/1) to afford 5-(4-(2-azidoethoxy)phenyl)-3-benzylpyrazin-2-amine (**15**) as a yellow solid (0.590 g, 68%):

^{1}H-NMR (300 MHz, $CDCl_3$) δ= 8.33 (s, 1H), 7.89 (d, *J* = 9 Hz, 2H), 7.19-7.38 (m, 5H), 7.01 (d, *J* = 8.7 Hz, 2H), 4.37 (s, 2H), 4.18-4.22 (m, 4H), 3.63 (t, *J* = 4.8 Hz, 2H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) = 41.8, 50.3, 67.1, 115.0, 127.2, 127.2, 128.7, 129.1, 130.8, 136.9, 137.0, 140.6, 142.5, 151.5, 158.5. HR-MS: m/z calcd for $C_{19}H_{19}N_6O_1$: 347.1616, found: 347.1620 $[M+H]^+$.

6-(4-(2-Azidoethoxy)phenyl)-8-benzyl-2-(4-hydroxybenzyl)imidazo[1,2-*a*]pyrazin-3(*7H*)-one (2) (6-N_3-CTZ)

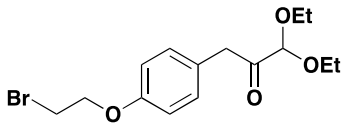


5-(4-(2-Azidoethoxy)phenyl)-3-benzylpyrazin-2-amine (**15**) (500 mg, 1.44 mmol, 1.0 eq.) and 3-(4-((tert-butyldimethylsilyl)oxy)phenyl)-1,1-diethoxypropan-2-one (**16**) (814 mg, 2.31 mmol, 1.6 eq.) were dissolved in ethanol (25 ml) and water (8 ml) and stirred at room temperature. After vacuum deaeration, the solution was cooled to 0 ℃ and HCl (4 ml) was added under nitrogen flow. Once the solution reached room temperature, it was heated and stirred for 15 hours at 80 ℃. The reaction solvent was evaporated and the crude compound was purified by means of silica gel column chromatography (eluent composition: ethyl acetate/methanol = 20/1) to afford 6-(4-(2-azidoethoxy)phenyl)-8-benzyl-2-(4-hydroxybenzyl)imidazo[1,2-*a*]pyrazin-3(*7H*)-one (**2**) (287 mg, 40%) as a yellow solid:

^{1}H-NMR (500 MHz, CD_3OD) δ= 7.55 (s, 1H), 7.41 (d, *J* = 7.5 Hz, 2H), 7.31 (t, *J* = 7.0 Hz, 2H), 7.23 (m, 5H), 7.04 (d, *J* = 9.0 Hz, 2H), 6.74 (d, *J* = 8.5 Hz, 2H), 4.39 (s, 2H), 4.22 (t, *J* = 5.0 Hz, 2H), 4.11 (s, 2H), 3.64 (t, *J* = 4.5 Hz, 2H). ^{13}C-NMR (125 MHz, CD_3OD, $CDCl_3$): δ (ppm) = 33.1, 35.1, 51.1, 68.2, 108.2, 127.6, 128.0, 129.1, 129.3,

129.6, 129.6, 129.7, 130.4, 130.5, 130.6, 137.8, 156.6, 160.6. HR-MS: m/z calcd for $C_{28}H_{25}N_6O_3$: 493.1970, found: 493.1988 $[M+H]^+$.

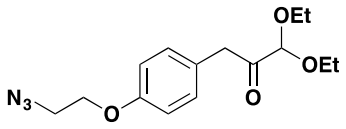
3-(4-(2-Bromoethoxy)phenyl)-1,1-diethoxypropan-2-one (20)



1,1-Diethoxy-3-(4-hydroxyphenyl)propan-2-one (**19**) (356.7 mg, 1.50 mmol, 1.0 eq.) and Cs_2CO_3 (635.3 mg, 1.95 mmol, 1.3 eq.) were dissolved in acetonitrile (6 ml) and stirred at room temperature. 1,2-dibromoethane (0.6 ml) was added into the solution and stirred at 100 ℃ overnight. After cooling to room temperature, the reaction mixture was evaporated and the residue was extracted with ethyl acetate, and the transparent organic phase was washed with water and brine, dried over sodium sulfate and evaporated. The resulting residue was purified by flash chromatography (silica gel, eluent composition: n-hexane/ethyl acetate = 9/1 to 8/2), affording 3-(4-(2-bromoethoxy)phenyl)-1,1-diethoxypropan-2-one (**20**) (147.7 mg, 29%) as a water-clear viscous oil:

^{1}H-NMR (500 MHz, $CDCl_3$) δ= 7.13 (d, *J* =8.3 Hz, 2H), 6.86 (d, *J* =8.5 Hz, 2H), 4.27 (t, *J* =6.3 Hz, 2H), 3.82 (s, 1H), 3.71-3.68 (m, 2H), 3.62 (t, *J* =6.3 Hz, 2H), 3.56-3.54 (m, 2H), 1.24 (t, *J* =7.1 Hz, 6H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) = 15.3, 29.3, 42.9, 63.5, 68.0, 102.5, 114.9, 126.7, 131.0, 157.2, 203.6.

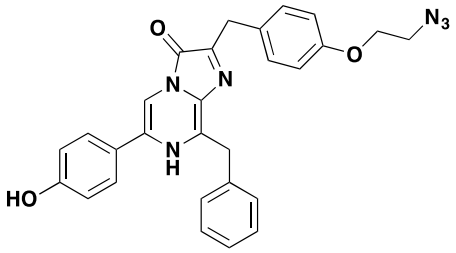
3-(4-(2-Azidoethoxy)phenyl)-1,1-diethoxypropan-2-one (21)



3-(4-(2-Bromoethoxy)phenyl)-1,1-diethoxypropan-2-one (**20**) (346.1 mg, 1.00 mmol, 1.0 eq.) was dissolved in DMF (9 ml). Sodium azide (80.0 mg, 1.18 mmol, 1.2 eq.) was added into the solution, followed by stirring for 2 hours at 100 ℃. After cooling to room temperature, the reaction mixture was evaporated and the residue was extracted with ethyl acetate, and the yellow organic phase was washed with water and brine, dried over sodium sulfate and evaporated, affording 3-(4-(2-Azidoethoxy)phenyl)-1,1-diethoxypropan-2-one (**21**) (300.1 mg, 98%) as a yellow-clear viscous oil:

^{1}H-NMR (500 MHz, $CDCl_3$) δ= 7.13 (d, *J* =8.5 Hz, 2H), 6.87 (d, *J* =8.5 Hz, 2H), 4.13 (t, *J* =5.1 Hz, 2H), 3.83 (s, 2H), 3.73-3.67 (m, 2H), 3.59-3.52 (m, 4H), 1.24 (t, *J* =6.3 Hz, 6H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) = 15.3, 42.9, 50.3, 63.5, 67.1, 102.4, 114.8, 126.6, 131.0, 157.3, 203.6.

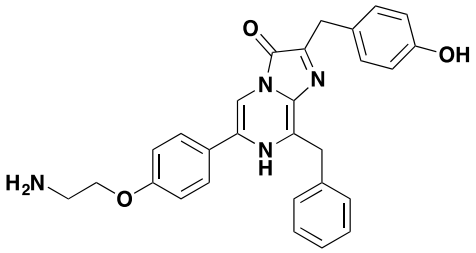
2-(4-(2-Azidoethoxy)benzyl)-8-benzyl-6-(4-hydroxyphenyl)imidazo[1,2-*a*]pyrazin-3(*7H*)-one (3) (2-N_3-CTZ)



4-(5-amino-6-benzylpyrazin-2-yl)phenol (**18**) (24.3 mg, 0.99 mmol, 1.0 eq.) and 3-(4-(2-azidoethoxy)phenyl)-1,1-diethoxypropan-2-one (**21**) (53.8 mg, 0.18 mmol, 2.0 eq.) were dissolved in ethanol (1.5 ml) and water (0.2 ml) and stirred at room temperature. After vacuum deaeration, the solution was cooled to 0 ℃ and HCl (0.1 ml) was added under nitrogen flow. Once the solution reached room temperature, it was heated and stirred for 18 hours at 80 ℃. The reaction solvent was evaporated and the crude compound was purified by means of preparative HPLC (eluent composition: acetonitrile/water = 1/1) to afford 2-(4-(2-azidoethoxy)phenyl)-8-benzyl-6-(4-hydroxybenzyl)imidazo[1,2-*a*]pyrazin-3(*7H*)-one (**3**) (41 mg, 48%) as a yellow solid:

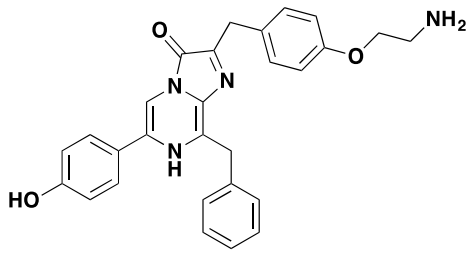
^{1}H-NMR (300 MHz, $CDCl_3$) δ= 7.44-7.35 (m, 4H), 7.29-7.19 (m, 5H), 6.87-6.83 (m, 4H), 4.38 (s, 2H), 4.11-4.09 (m, 4H), 3.53 (t, *J* = 4.9 Hz, 2H).

6-(4-(2-Aminoethoxy)phenyl)-8-benzyl-2-(4-hydroxybenzyl)imidazo[1,2-a]pyrazin-3(7H)-one (22) (6-NH_2-CTZ)



6-N_3-CTZ (**2**) (20 mg, 0.04 mmol, 1.0 eq.) was dissolved in methanol (5 ml). After vacuum deaeration, a catalytic amount of 5% Pd/C was added into the solution and the mixture was deaerated again and stirred for 5 hours at 40 ℃ under H_2 atmosphere. The solution was filtered through a Celite pad to remove the catalyst. The solution was evaporated to afford crude 6-(4-(2-aminoethoxy)phenyl)-8-benzyl-2-(4-hydroxybenzyl)imidazo[1,2-a]pyrazin-3(7H)-one (**22**), which was used directly for the next reaction: HR-MS: m/z calcd for $C_{28}H_{27}N_4O_3$: 467.2083, found: 467.2095 $[M+H]^+$.

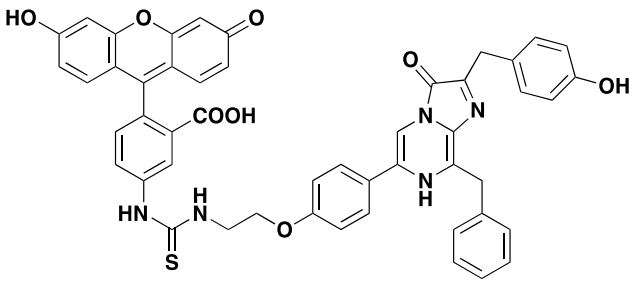
2-(4-(2-Aminoethoxy)benzyl)-8-benzyl-6-(4-hydroxyphenyl)imidazo[1,2-*a*]pyrazin-3(*7H*)-one (23) (2-NH_2-CTZ)



2-N_3-CTZ (**3**) (106.0 mg, 0.21 mmol, 1.0 eq.) was dissolved in methanol (8 ml). After vacuum deaeration, a catalytic amount of 5% Pd/C was added into the solution and the mixture was deaerated again and stirred for 15 hours at 40 ℃ under H_2 atmosphere. The

solution was filtered through a Celite pad to remove the catalyst. The solution was evaporated to afford crude 2-(4-(2-aminoethoxy)benzyl)-8-benzyl-6-(4-hydroxyphenyl)imidazo[1,2-a]pyrazin-3(7H)-one (**23**), which was used directly for the next reaction.

5-(3-(2-(4-(8-Benzyl-2-(4-hydroxybenzyl)-3-oxo-3,7-dihydroimidazo[1,2-a]pyrazin-6-yl)phenoxy)ethyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (4) (6-FITC-CTZ)



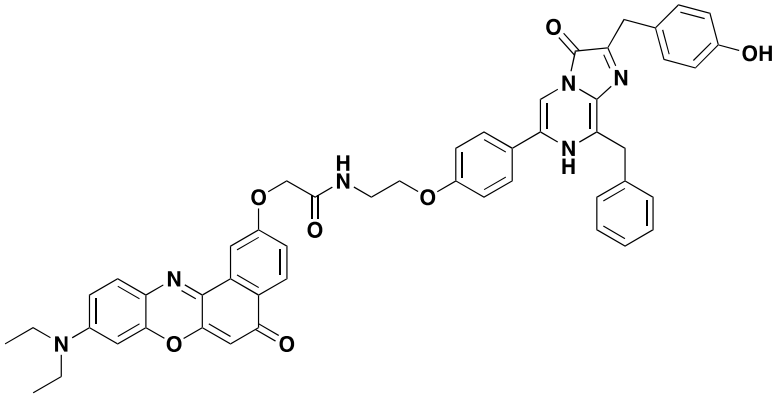
Fluorescein isothiocyanate (7.8 mg, 0.02 mmol, 1.0 eq.) and 6-NH_2-CTZ (**15**) (20 mg, 0.04 mmol, 2.0 eq.) were dissolved in ethanol (5 ml) and tetrahydrofuran (3 ml) and stirred for 3 hours at 40 ℃. The reaction solvent was evaporated and the crude compound was purified by means of preparative HPLC (eluent composition: acetonitrile/water =

2/1) to afford 5-(3-(2-(4-(8-benzyl-2-(4-hydroxybenzyl)-3-oxo-3,7-dihydroimidazo[1,2-a]pyrazin-6-yl)phenoxy)ethyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (**4**) (2.2 mg, 13%) as a yellow solid:

^{1}H-NMR (500 MHz, CD_3OD) δ= 8.11 (d, *J* = 2.0 Hz, 1H), 7.71 (d, *J* = 7.0 Hz, 1H), 7.59 (s, 2H), 7.38 (d, *J* = 7.5 Hz, 2H), 7.28 (t, *J* = 7.0 Hz, 2H), 7.22 (t, *J* = 7.5 Hz, 1H), 7.14 (m, 5H), 6.68 (m, 6H), 6.54 (q, *J* = 8.5 Hz, 2H), 4.40 (s, 2H), 4.30 (t, *J* = 5.5 Hz, 2H), 4.60 (m, 4H). HR-MS: m/z calculated for $C_{49}H_{38}N_5O_8S$: 856.2441, found: 856.2430 $[M+H]^+$.

In the synthesis process of 6-FITC-CTZ, the intermediate 6-NH_2-CTZ has the potential to cause artifact effects in the spectra. However, this concern is excluded by the fact that the hydrogens of 6-FITC-CTZ show singlet peaks at the δ = 4 - 4.5 region of the H^1 NMR spectrum, implicating the high purity.

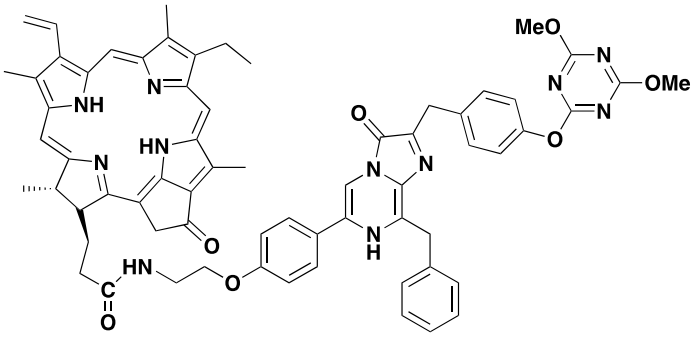
N-(2-(4-(8-benzyl-2-(4-hydroxybenzyl)-3-oxo-3,7-dihydroimidazo[1,2-a]pyrazin-6-yl)phenoxy)ethyl)-2-((9-(diethylamino)-5-oxo-5H-benzo[a]phenoxazin-2-yl)oxy)acetamide (5) (6-Nile-Red-CTZ)



2,5-Dioxopyrrolidin-1-yl2-((9-(diethylamino)-5-oxo-5H-benzo[a]phenoxazin-2-yl)oxy)acetate (24.0 mg, 0.04 mmol, 1.0 eq.) and 6-NH_2-CTZ (**15**) (45.7 mg, 0.09 mmol, 2.0 eq.) were dissolved in methanol (0.5 ml) and tetrahydrofuran (3 ml) and stirred for 30 minutes at 60 ℃. The reaction solvent was evaporated and the crude compound was purified by means of silica column chromatography (eluent composition: chloroform/methanol = 19/1) to afford N-(2-(4-(8-benzyl-2-(4-hydroxybenzyl)-3-oxo-3,7-dihydroimidazo[1,2-a]pyrazin-6-yl)phenoxy)ethyl)-2-((9-(diethylamino)-5-oxo-5H-benzo[a]phenoxazin-2-yl)oxy)acetamide (**5**) (17.8 mg, 43%) as a purple solid:

^{1}H-NMR (300 MHz, DMSO-d_6) δ=8.08 (d, *J* =8.8 Hz, 1H), 7.95 (d, *J* =2.4 Hz, 1H), 7.46 (d, *J* =7.2 Hz, 2H), 7.39 (d, *J* =9.2 Hz, 2H), 7.33 (t, *J* =6.8 Hz, 3H), 7.24 (m, 6H), 6.74 (d, *J* =8.8 Hz, 2H), 6.66 (dd, *J* =2.4 Hz, 8.8 Hz, 1H), 6.60 (d, *J* =8.8 Hz, 2H), 6.17 (s, 1H), 6.12 (s, 1H), 4.72 (s, 2H), 4.38 (s, 2H), 4.09 (s, 2H), 4.02 (t, *J* =6.4 Hz, 1H), 3.65 (t, *J* =6.4 Hz, 3H), 3.21 (q, *J* =7.2 Hz, 4H), 1.07 (t, *J* =7.2 Hz, 6H). HR-MS: m/z calcd for $C_{50}H_{45}N_6O_7$: 841.3350, found: 841.3351 $[M+H]^+$.

6-Chlorin-2-DMT-CTZ (6)

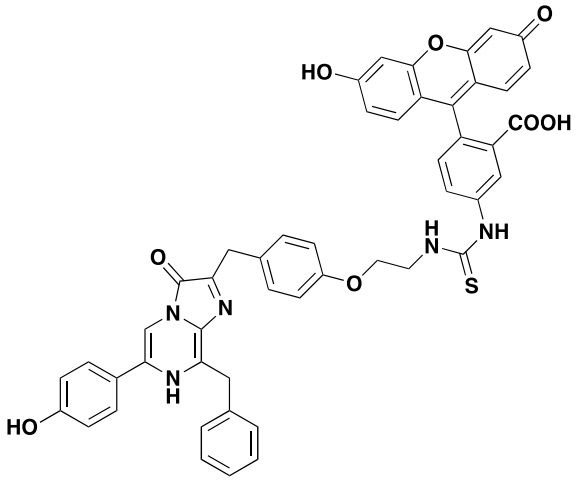


Pyropheophorbide (10.9 mg, 0.02 mol, 1.0 eq.) and 4(- 4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride n-hydrate (11.0 mg, 0.04 mmol, 2.0 eq.) were dissolved in THF (0.5 mL) stirred for 30 minutes at 60 ℃. 6-NH_2-CTZ (**15**) (9.3 mg, 0.02 mmol, 1.0 eq.) dissolved in methanol (1 mL) was added into the reaction mixture and stirred at 60 ℃ overnight. The reaction solvent was evaporated, and the crude compound was purified by chromatograph on a silicagel plate (eluent composition: ethyl acetate/methanol = 9/1) to afford 6-Chlorin-2-DMT-CTZ (6) (10.0 mg, 51%) as a brown solid:

^{1}H-NMR (400 MHz, $CDCl_3$) δ= 9.39 (s, 1H), 9.35 (s, 1H), 8.53 (s, 1H), 7.97 (dd, *J* =11.6 Hz, 18 Hz, 1H), 7.71 (s, 1H), 7.62 (d, *J* = 8.8 Hz, 2H), 7.53 (d, *J* =7.2 Hz, 2H), 7.22 (m, 5H), 7.00 (d, *J* =8.4 Hz, 2H), 6.62 (d, *J* =8.4 Hz, 2H), 6.57 (d, *J* =8.4 Hz, 2H), 6.26 (d, *J* =17.6 Hz, 1H), 6.14 (d, *J* =11.6 Hz, 1H), 5.50 (t, *J* =5.5 Hz, 1H), 5.22 (d, *J* =20 Hz, 1H), 5.06 (d, *J* =20 Hz, 1H), 4.88 (q, *J* =7.5 Hz, 1H), 4.54 (s, 2H), 4.33 (s, 1H), 4.01 (m,

2H), 3.95 (s, 6H), 3.81 (s, 1H), 3.51 (s, 3H), 3.38 (s, 4H), 3.21 (s, 3H), 2.64 (m, 3H), 2.45 (m, 1H), 2.24 (m, 1H), 1.90 (m, 1H) 1.80 (d, *J* =7.2 Hz, 3H), 1.66 (t, *J* =8.0 Hz, 6H). HR-MS: m/z calcd for $C_{66}H_{64}N_{11}O_7$: 1122.4990, found: 1122.4983 $[M+H]^+$.

5-(3-(2-(4-(8-Benzyl-6-(4-hydroxyphenyl)-3-oxo-3,7-dihydroimidazo[1,2-a]pyrazin-6-yl)methyl)phenoxy)ethyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (8) (2-FITC-CTZ)

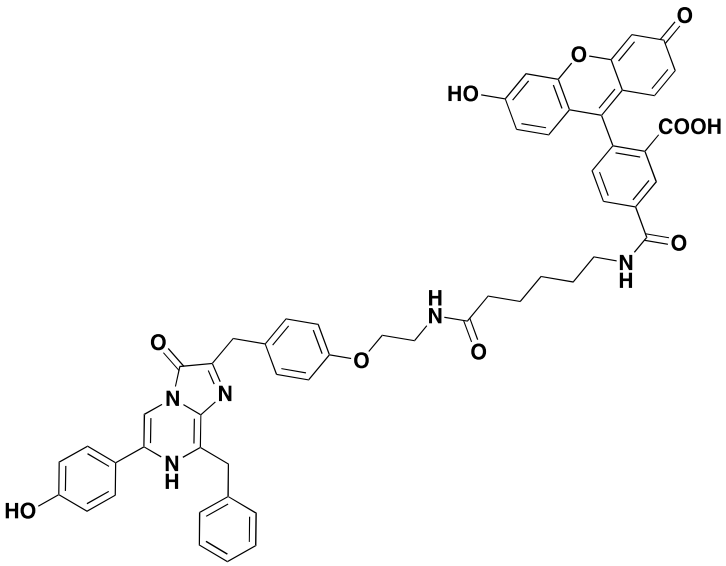


Fluorescein isothiocyanate (9.8 mg, 0.02 mmol, 0.8 eq.) and 2-NH_2-CTZ (**16**) (14.2 mg, 0.03 mmol, 1.0 eq.) were dissolved in tetrahydrofuran (5 ml) and stirred for 2 hours at room temperature. The reaction solvent was evaporated and the crude compound was purified by means of preparative HPLC (eluent composition: acetonitrile/water = 1/1) to afford 5-(3-(2-(4-(8-benzyl-6-(4-hydroxyphenyl)-3-oxo-3,7-dihydroimidazo[1,2-

a]pyrazin-6-yl)methyl)phenoxy)ethyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (**8**) (4.3 mg, 17%) as a yellow solid:

^{1}H-NMR (500 MHz, CD_3OD) δ= 8.13 (d, *J* = 1.8 Hz, 1H), 7.72 (dd, *J* = 8.3 Hz, 2.0 Hz, 1H), 7.44-7.36 (m, 4H), 7.29-7.20 (m, 5H), 7.12 (d, *J* = 8.3 Hz, 1H), 6.92-6.87 (m, 4H), 6.66-6.64 (m, 4H), 6.52 (dd, *J* = 8.7 Hz, 2.3 Hz, 2H), 4.38 (s, 2H), 4.20 (t, *J* = 5.2 Hz, 2H), 4.10 (s, 2H), 4.00 (t, *J* = 4.9 Hz, 2H).

5-((6-((2-(4-((8-Benzyl-6-(4-hydroxyphenyl)-3-oxo-3,7-dihydroimidazo[1,2-a]pyrazin-2-yl)methyl)phenoxy)ethyl)amino)-6-oxohexyl)carbamoyl)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid (9) (2-SFX-CTZ)

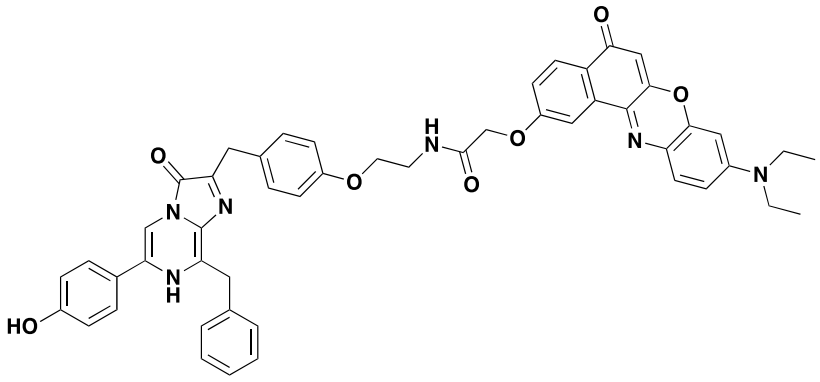


5-Fluorescein succinimidyl ester (5.0 mg, 0.008 mmol, 1.0 eq.) and 2-NH_2-CTZ (**16**) (30.1 mg, 0.06 mmol, 7.5 eq.) were dissolved in methanol (2 ml) and tetrahydrofuran (2 ml) and stirred for 2 hours at 40 ℃. The reaction solvent was evaporated and the crude compound was purified by means of preparative HPLC (eluent composition: acetonitrile/water = 1/1) to afford 5-((6-((2-(4-((8-Benzyl-6-(4-hydroxyphenyl)-3-oxo-3,7-dihydroimidazo[1,2-a]pyrazin-2-yl)methyl)phenoxy)ethyl)amino)-6-oxohexyl)carbamoyl)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid (**9**) (1.7 mg, 21%) as an orange-brown solid:

^{1}H-NMR (500 MHz, CD_3OD) δ= 8.40 (d, *J* = 1.2 Hz, 1H), 8.25 (bs, 2H), 8.15 (dd, *J* = 8.0 Hz, 1.7 Hz, 1H), 7.44-7.35 (m, 4H), 7.29-7.21 (m, 6H), 6.87 (d, *J* = 8.6 Hz, 2H), 6.82 (d, *J* = 8.6 Hz, 2H), 6.66 (d, *J* = 2.3 Hz, 2H), 6.54-6.48 (m, 4H), 4.37 (s, 2H), 4.07 (s, 2H),

3.99 (t, *J* = 5.2 Hz, 2H), 3.53 (t, *J* = 5.2 Hz, 2H), 3.36 (t, *J* = 6.9 Hz, 2H), 2.23 (t, *J* = 7.2 Hz, 2H), 1.64 (sep, *J* = 7.5 Hz, 2H), 1.39 (m, 2H).

<u>*N*-(2-(4-((8-benzyl-6-(4-hydroxyphenyl)-3-oxo-3,7-dihydroimidazo[1,2-*a*]pyrazin-2-yl)methyl)phenoxy)ethyl)-2-((9-(diethylamino)-5-oxo-5*H*-benzo[a]phenoxazin-2-yl)oxy)acetamide (10) (2-Nile-Red-CTZ)</u>

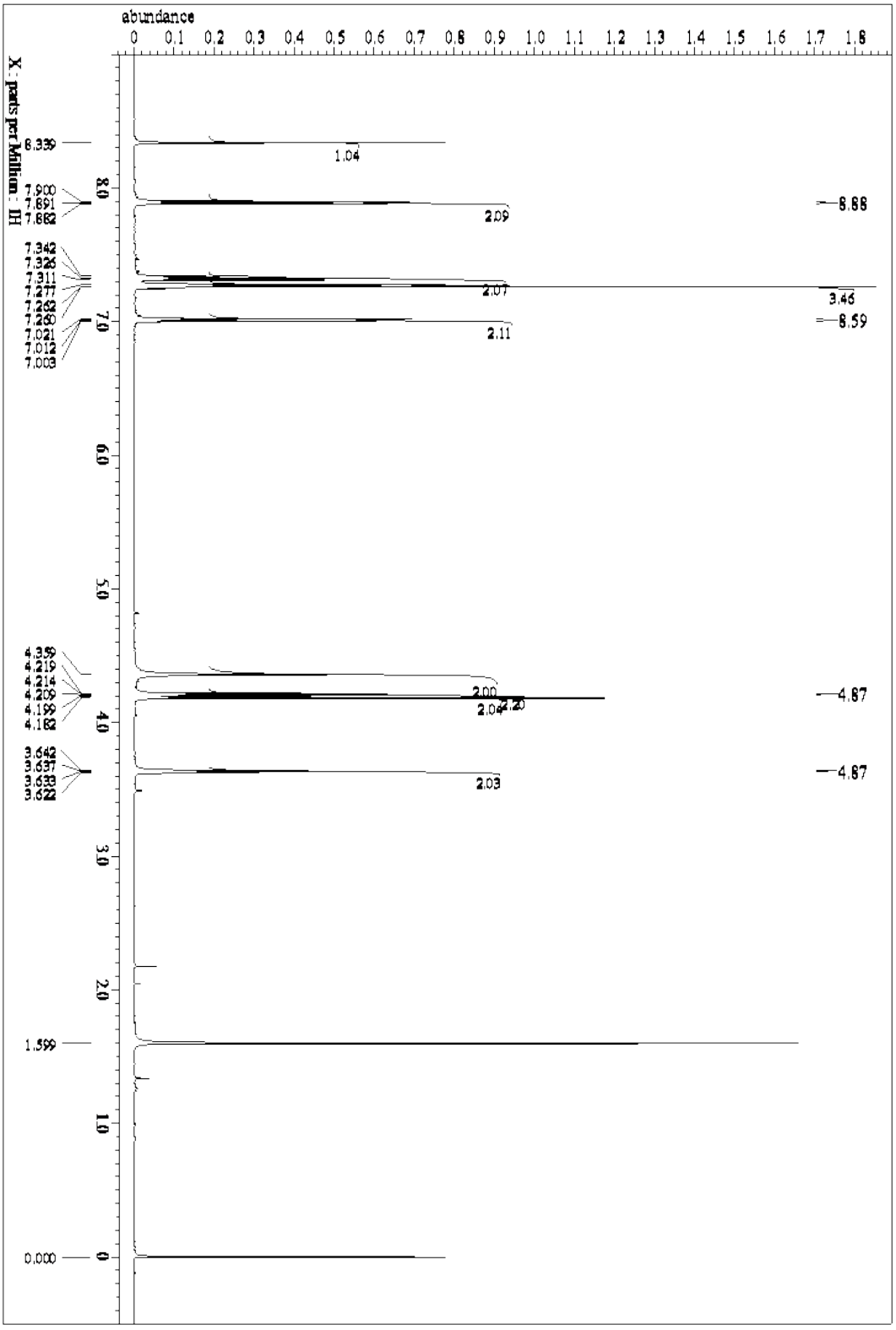


2,5-Dioxopyrrolidin-1-yl2-((9-(diethylamino)-5-oxo-5H-benzo[a]phenoxazin-2-yl)oxy)acetate (30.8 mg, 0.06 mmol, 1.8 eq.) and 2-NH_2-CTZ (**16**) (16.1 mg, 0.03 mmol, 1.0 eq.) were dissolved in methanol (3.0 ml) and stirred for 30 minutes at 40 ℃. The reaction solvent was evaporated and the crude compound was purified by means of silica column chromatography (eluent composition: chloroform/methanol = 19/1 to 9/1) to afford *N*-(2-(4-((8-benzyl-6-(4-hydroxyphenyl)-3-oxo-3,7-dihydroimidazo[1,2-*a*]pyrazin-2-yl)methyl)phenoxy)ethyl)-2-((9-(diethylamino)-5-oxo-5*H*-benzo[a]phenoxazin-2-yl)oxy)acetamide **(10)** (4.7 mg, 16%) as a purple solid:

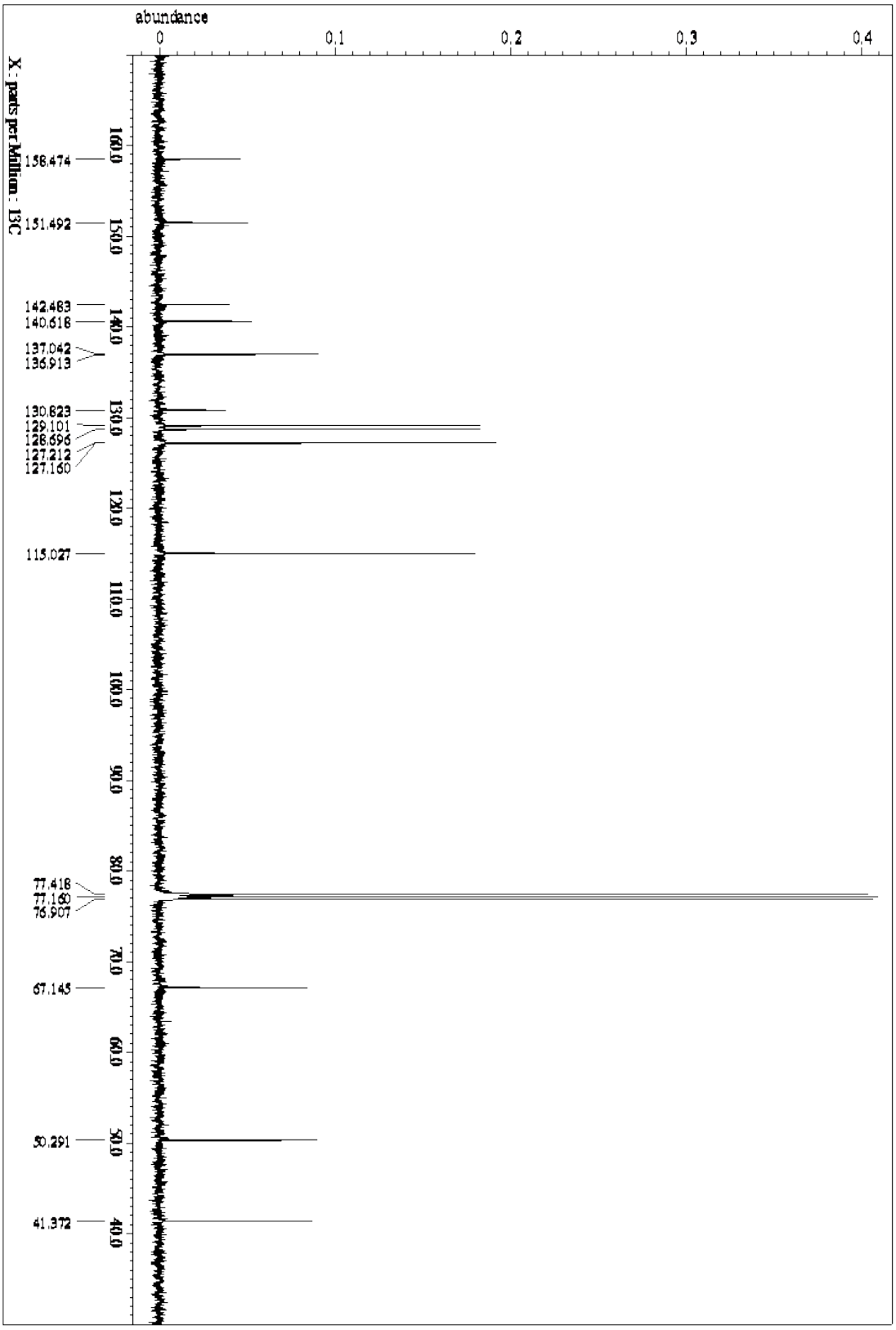
^{1}H-NMR (500 MHz, CD_3OD, $CDCl_3$) δ= 8.34 (s, 1H), 8.09 (d, *J* =8.6 Hz, 1H), 7.95 (d, *J* =2.6 Hz, 1H), 6.81 (d, *J* =9.2 Hz, 1H), 7.37-7.20 (m, 8H), 7.07 (d, *J* =7.7 Hz, 2H), 6.85 (d, *J* =8.6 Hz, 2H), 6.77 (dd, *J* =9.2 Hz, 2.6 Hz, 1H), 6.59 (d, *J* =8.6 Hz, 2H), 6.55 (s, 1H), 6.17 (s, 1H), 4.69 (s, 2H), 4.34 (s, 2H), 4.02 (s, 2H), 4.00 (t, *J* =4.9 Hz, 2H), 3.67 (t, *J* =4.9 Hz, 2H), 3.52 (q, *J* =7.2 Hz, 4H), 1.26 (t, *J* =7.3 Hz, 6H).

NMR characterization of synthesized compounds

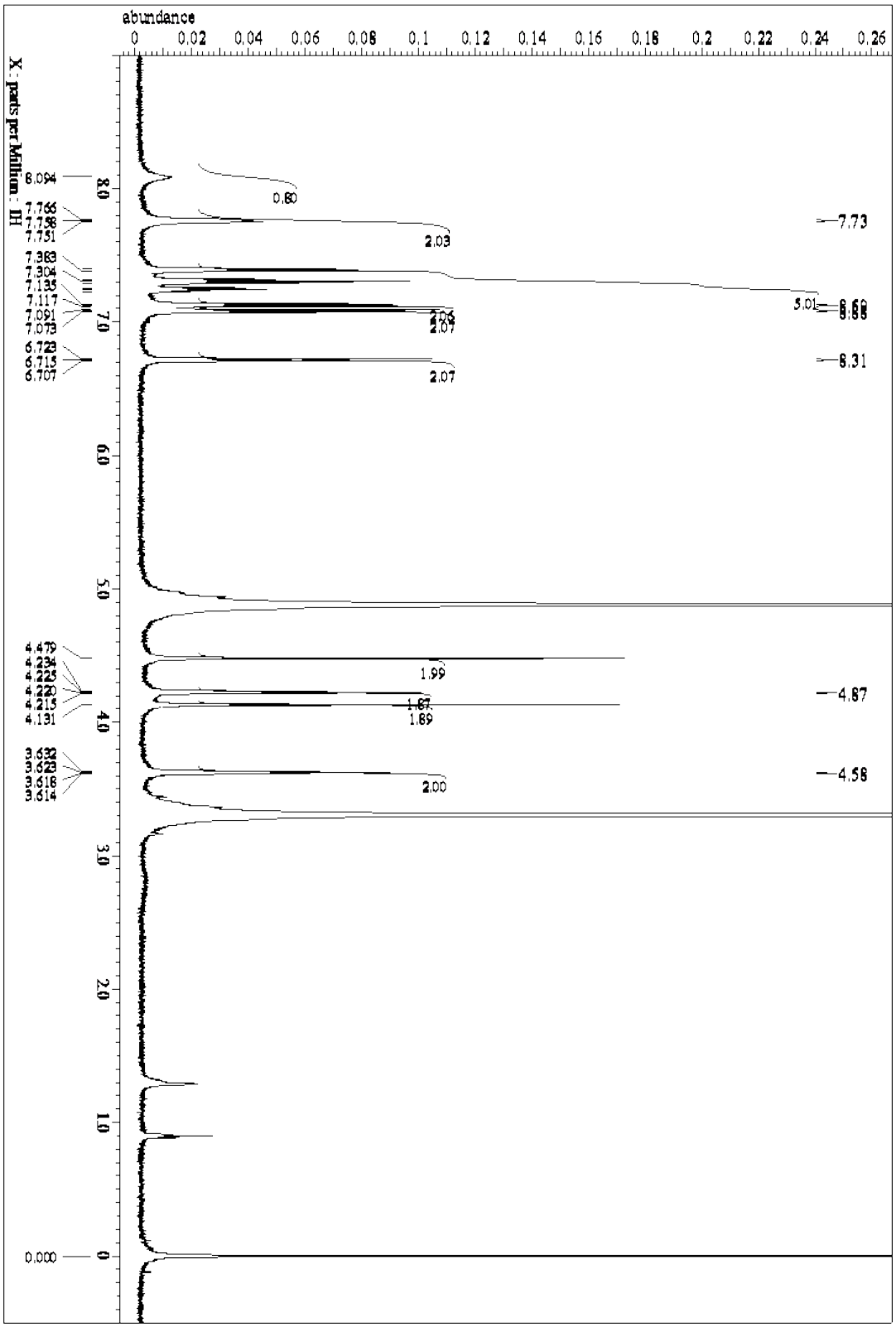
^{1}H NMR 5-(4-(2-Azidoethoxy)phenyl)-3-benzylpyrazin-2-amine (15)


abundance
X : parts per Million : 1H
8.339
7.900
7.891
7.882
7.342
7.326
7.311
7.277
7.262
7.260
7.021
7.012
7.003
4.359
4.219
4.214
4.209
4.199
4.182
3.642
3.637
3.633
3.622
1.599
0.000
1.04
2.09
2.07
2.11
2.00
2.04
2.03
8.88
3.46
8.59
4.87
4.87

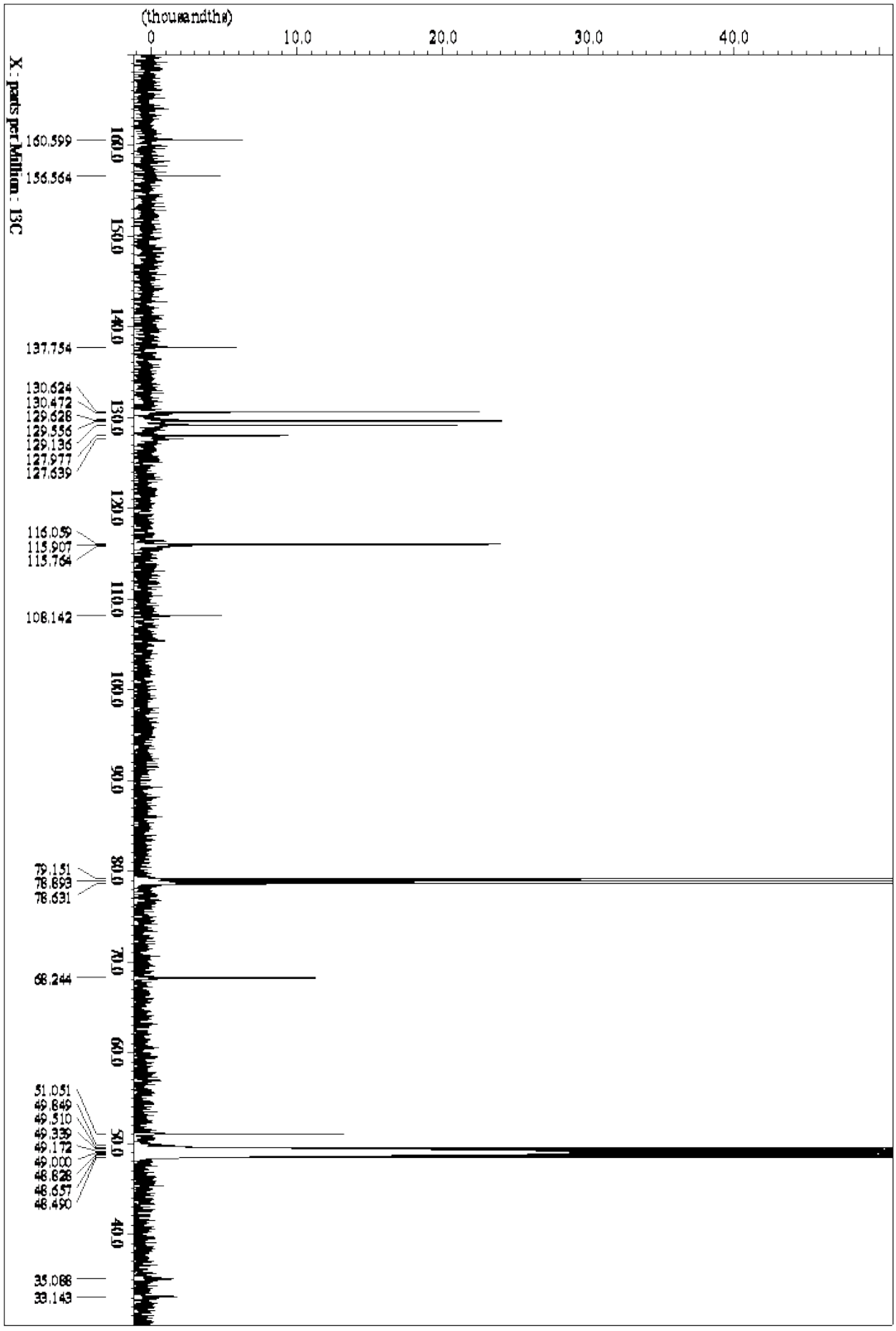
^{13}C NMR 5-(4-(2-Azidoethoxy)phenyl)-3-benzylpyrazin-2-amine (15)



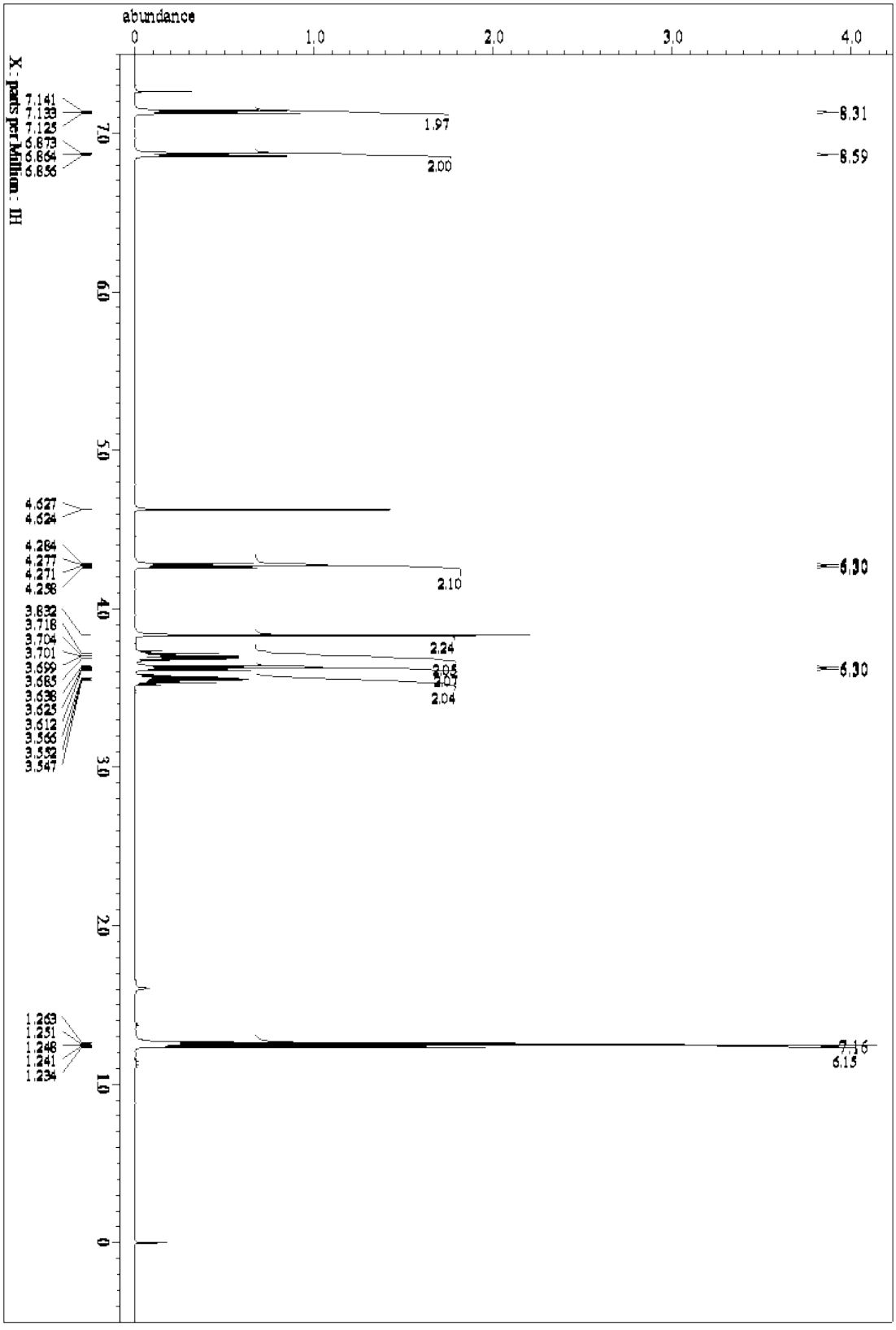
^{1}H NMR 6-(4-(2-Azidoethoxy)phenyl)-8-benzyl-2-(4-hydroxybenzyl)imidazo[1,2-*a*]pyrazin-3(*7H*)-one (2) (6-N_3-CTZ)


abundance
X : parts per Million : 1H
8.094
7.766
7.758
7.751
7.383
7.304
7.135
7.117
7.091
7.073
6.723
6.715
6.707
4.479
4.234
4.225
4.220
4.215
4.191
3.632
3.623
3.618
3.614
0.000
0.80
2.03
2.07
2.07
1.99
1.89
2.00
7.73
5.01
8.31
4.87
4.58

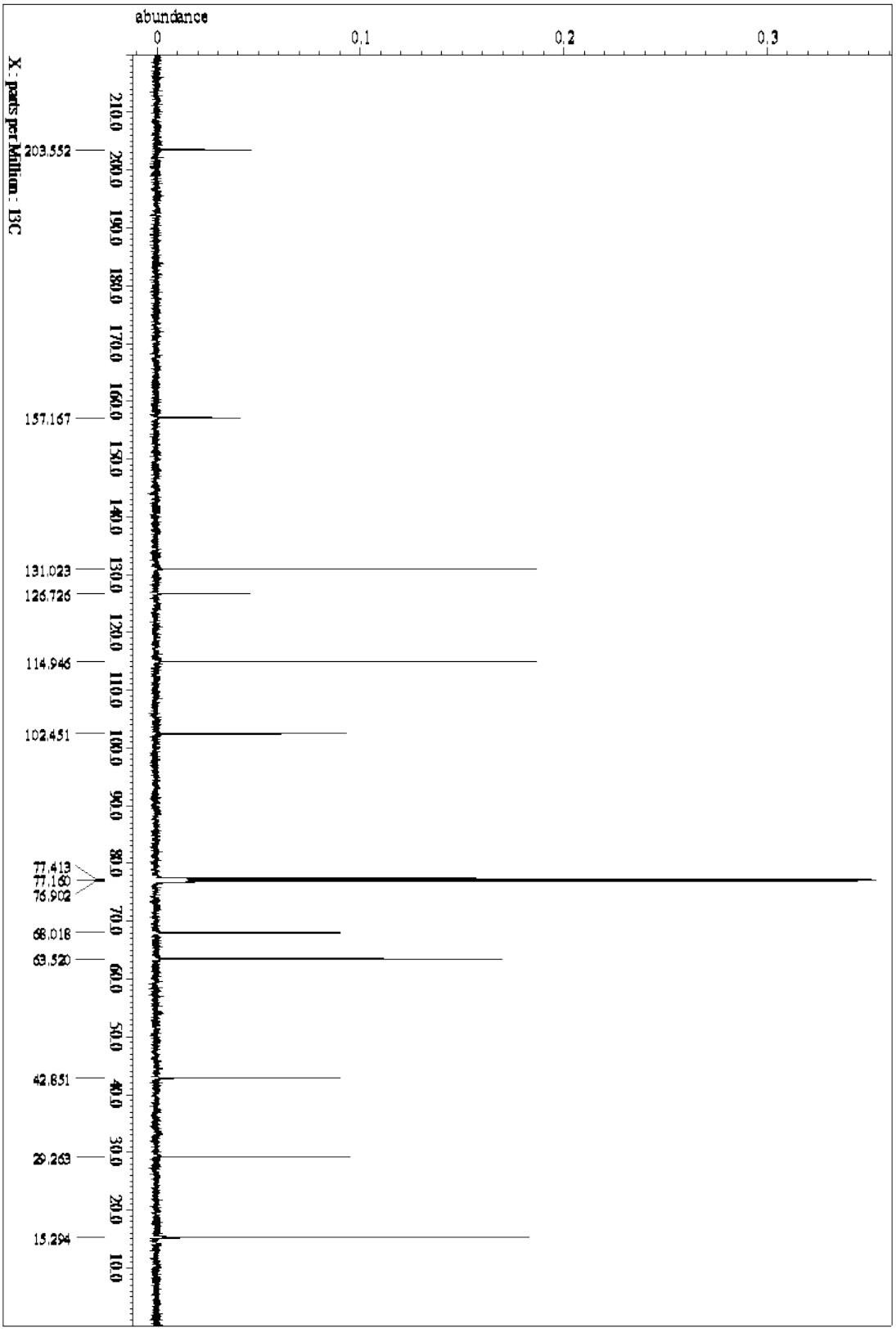
^{13}C NMR 6-(4-(2-Azidoethoxy)phenyl)-8-benzyl-2-(4-hydroxybenzyl)imidazo[1,2-*a*]pyrazin-3(*7H*)-one (2) (6-N_3-CTZ)



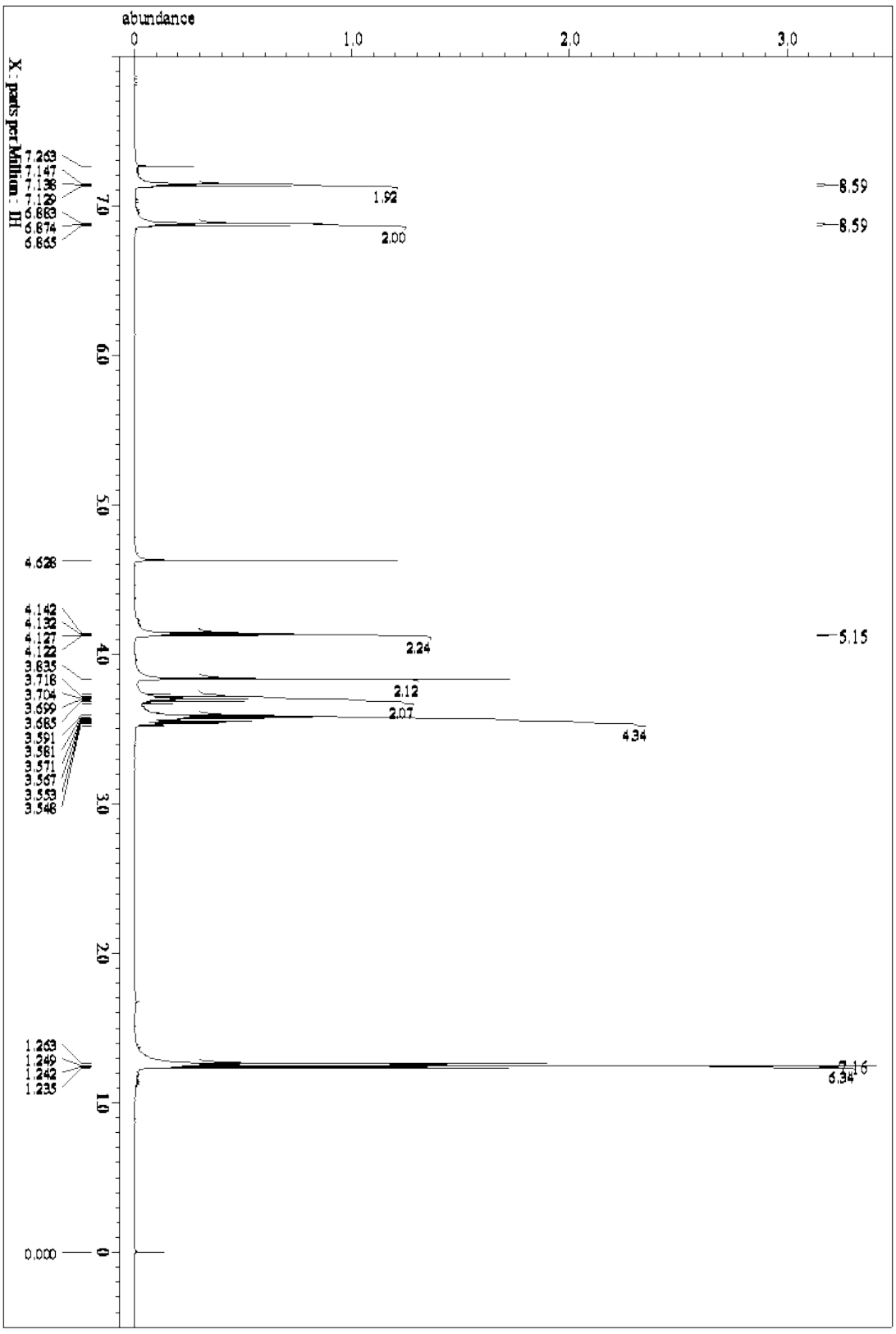
^{1}H NMR 3-(4-(2-bromoethoxy)phenyl)-1,1-diethoxypropan-2-one (20)



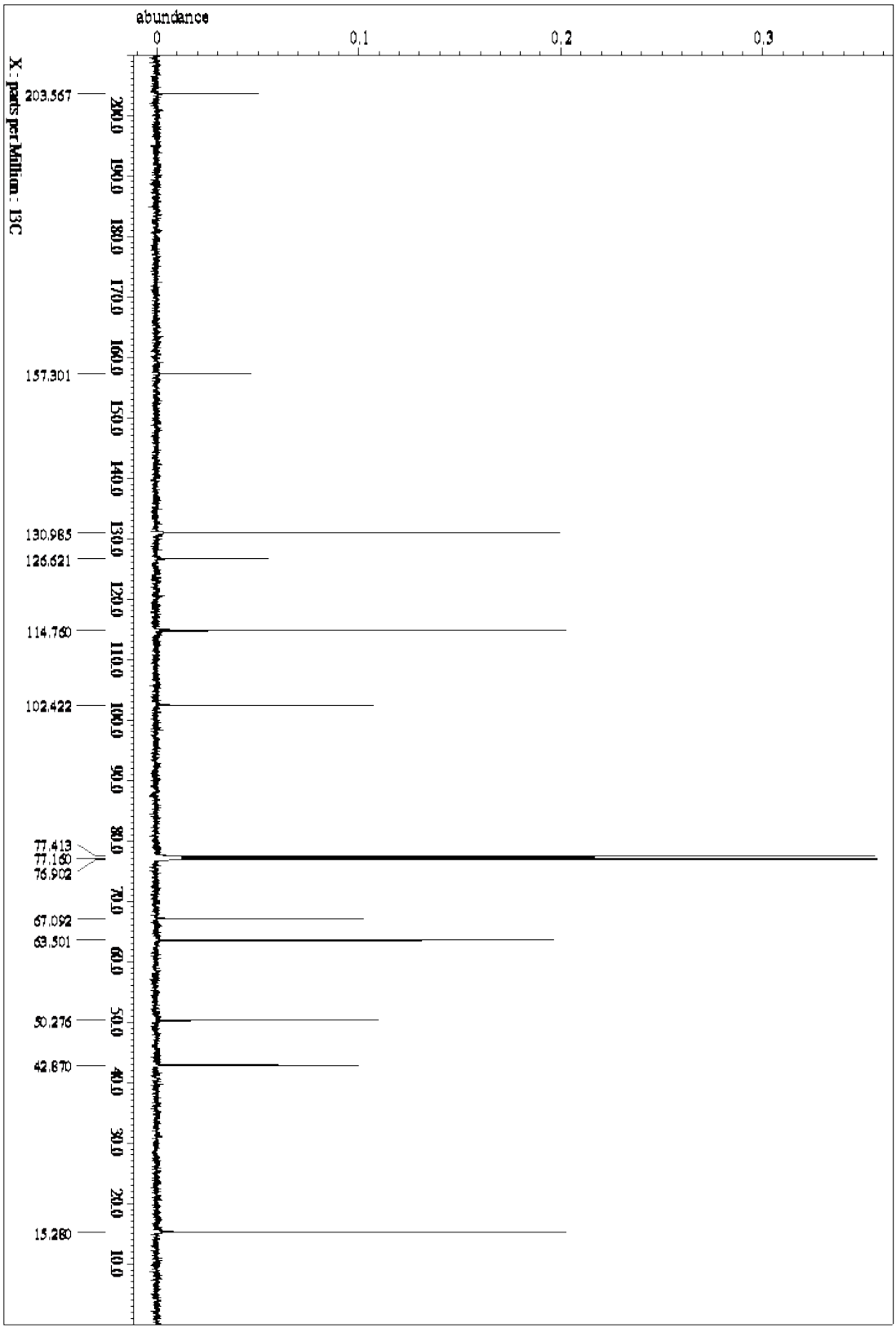
[13]C NMR 3-(4-(2-bromoethoxy)phenyl)-1,1-diethoxypropan-2-one (20)



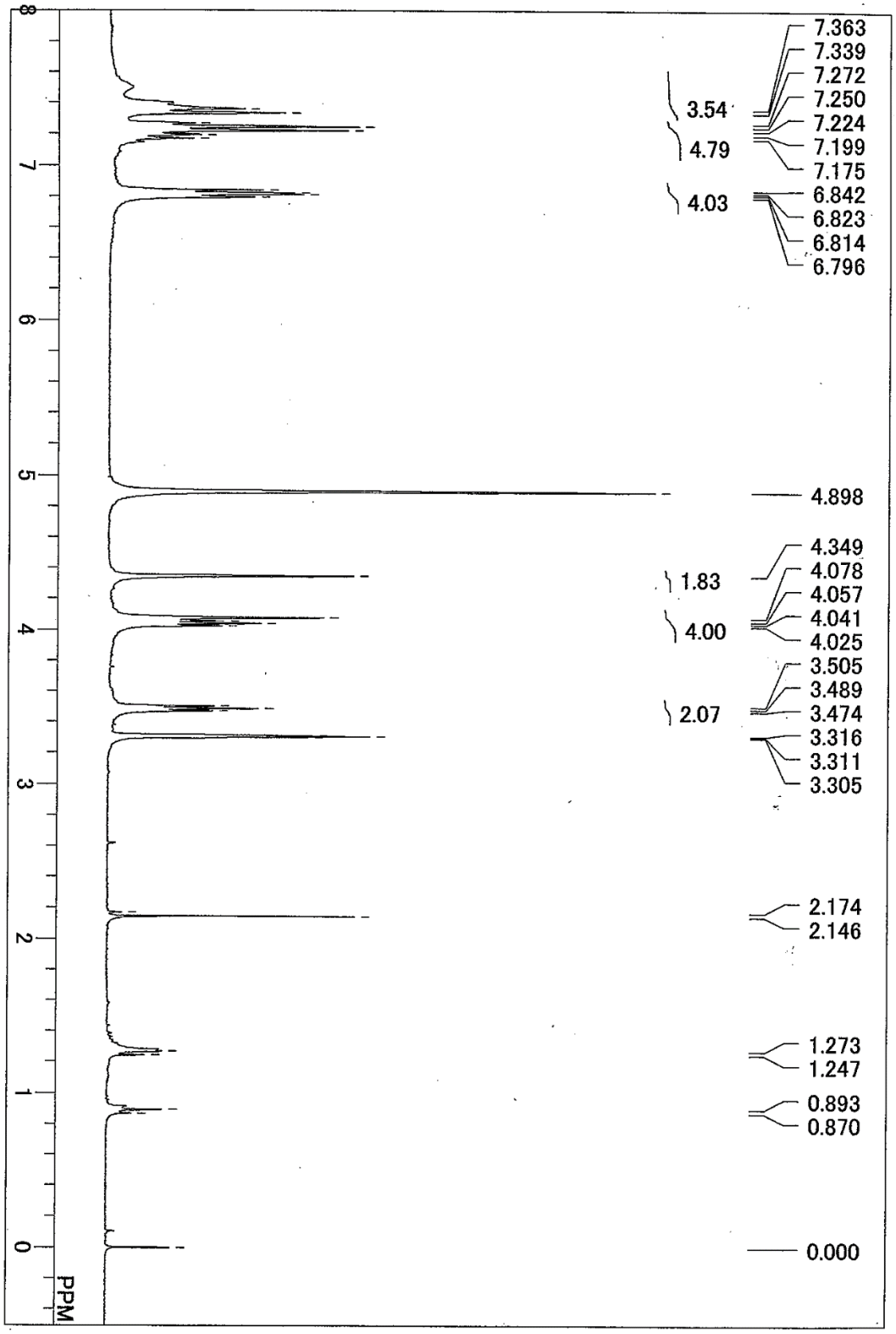
^{1}H NMR 3-(4-(2-azidoethoxy)phenyl)-1,1-diethoxypropan-2-one (21)



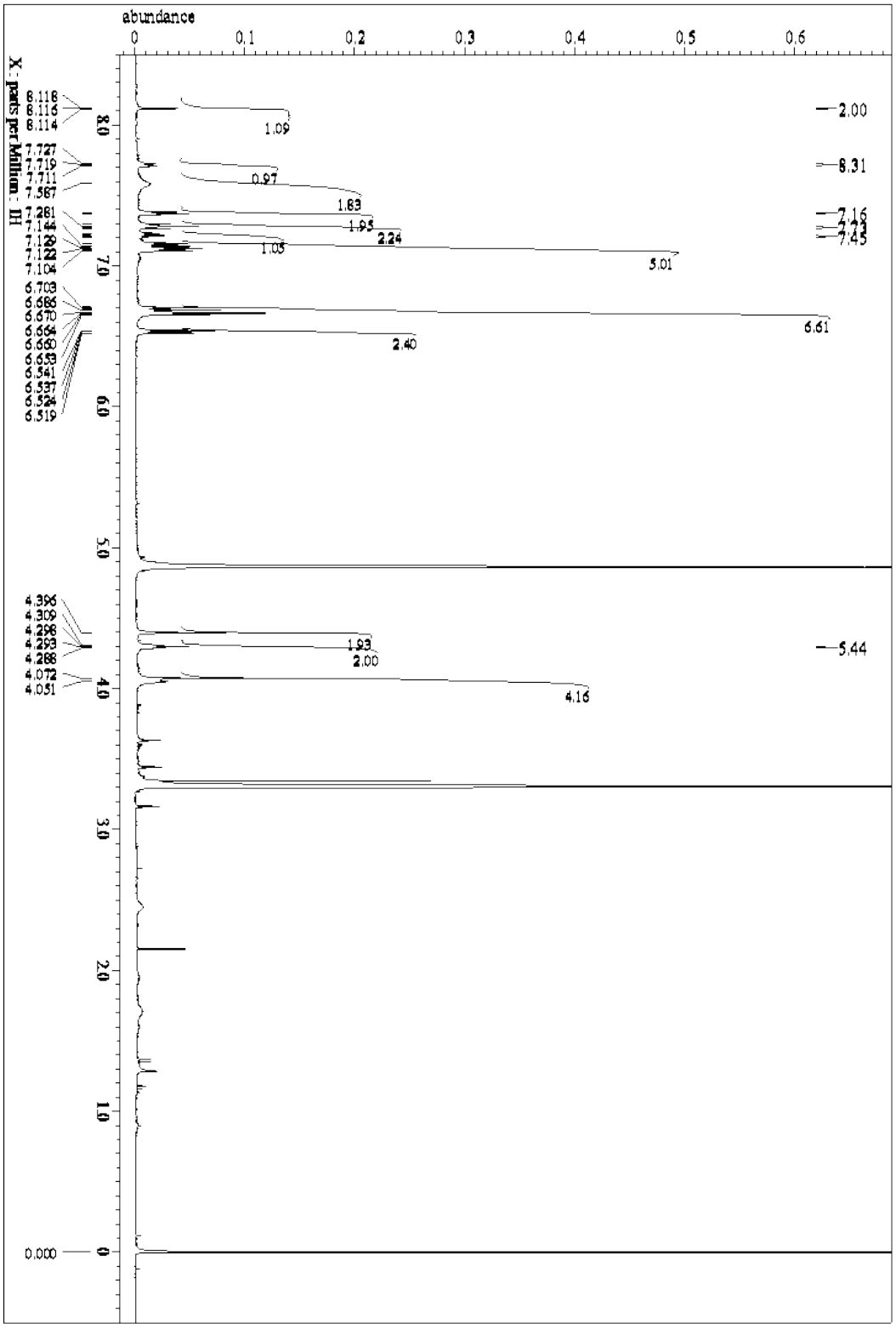
[13]C NMR 3-(4-(2-azidoethoxy)phenyl)-1,1-diethoxypropan-2-one (21)


abundance
0
0.1
0.2
0.3
X : parts per Million : 13C
203.567
157.301
130.985
126.621
114.760
102.422
77.413
77.160
76.902
67.092
63.501
50.276
42.670
15.280
200.0
190.0
180.0
170.0
160.0
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0

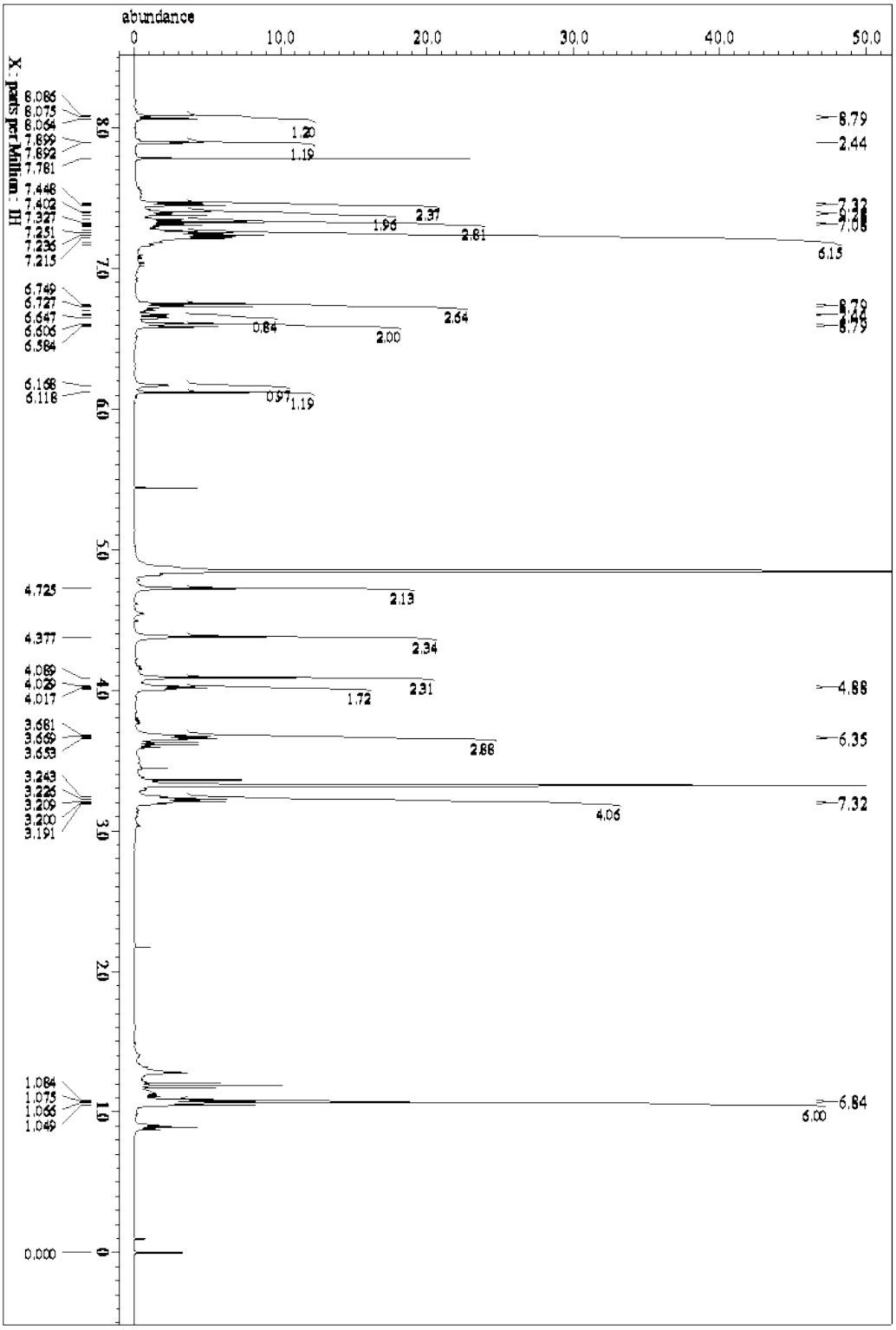
2-(4-(2-Azidoethoxy)benzyl)-8-benzyl-6-(4-hydroxyphenyl)imidazo[1,2-*a*]pyrazin-3(*7H*)-one (3) (2-N_3-CTZ)



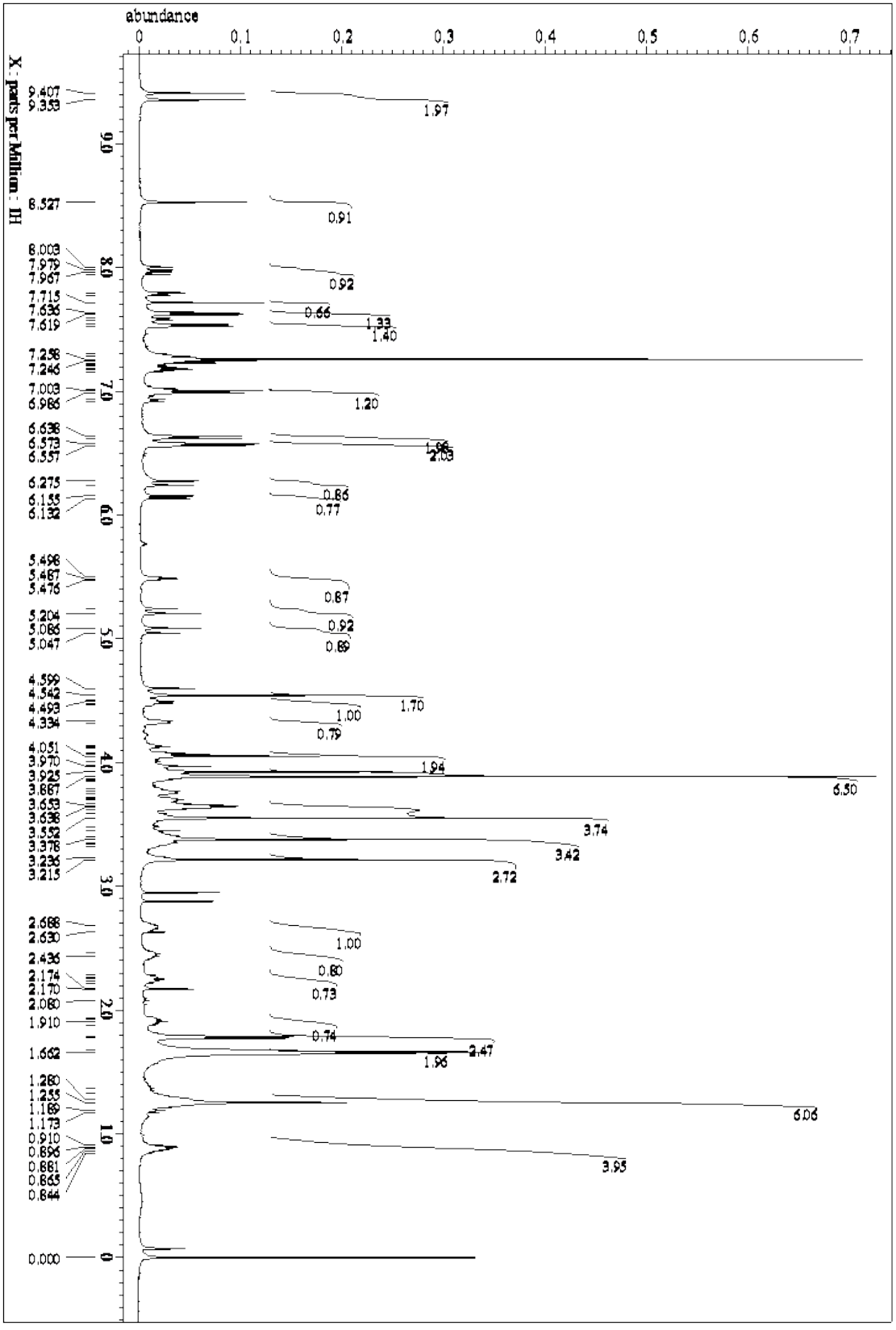
^{1}H NMR 5-(3-(2-(4-(8-Benzyl-2-(4-hydroxybenzyl)-3-oxo-3,7-dihydroimidazo[1,2-a]pyrazin-6-yl)phenoxy)ethyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (4) (6-FITC-CTZ)



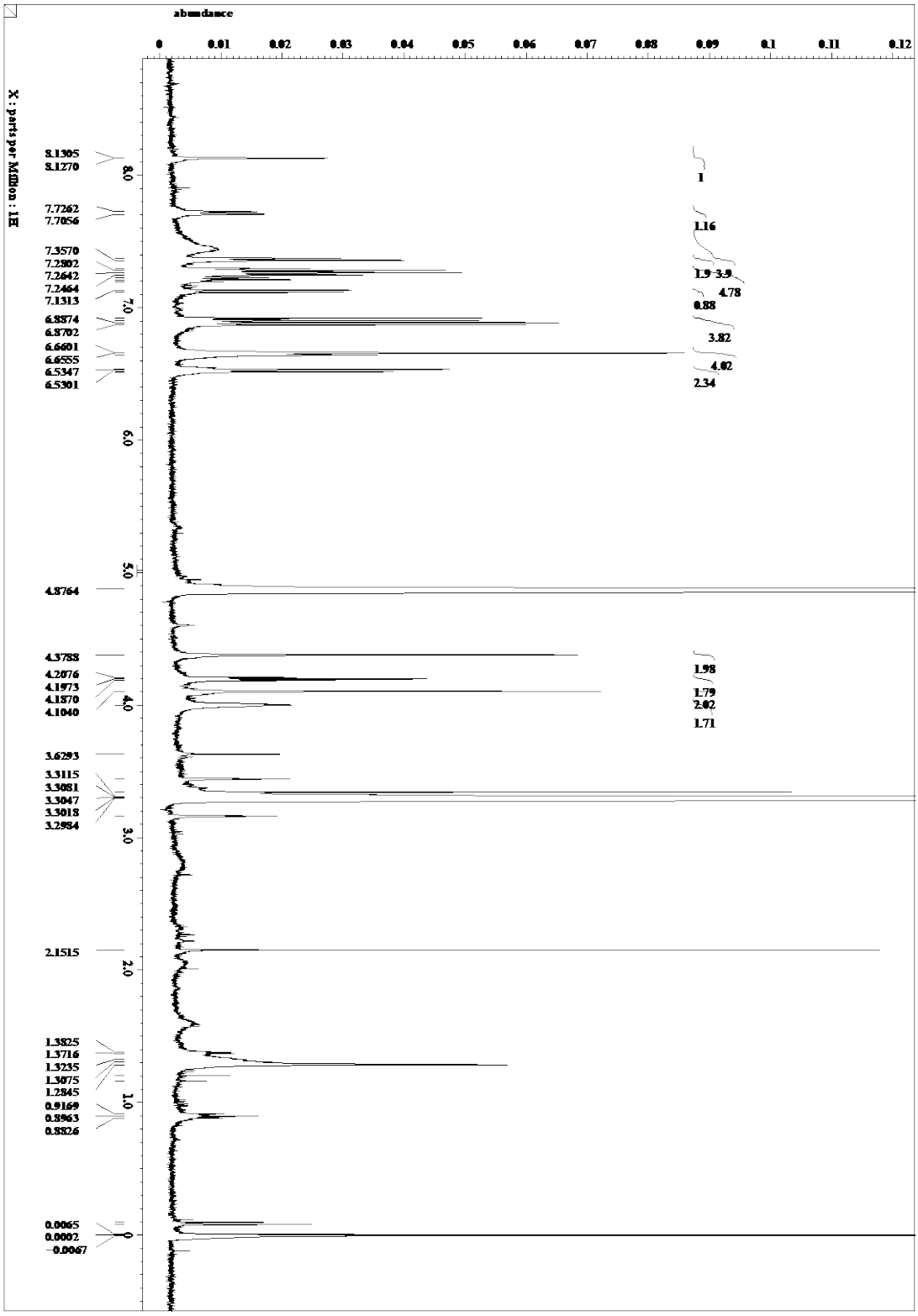
N-(2-(4-(8-benzyl-2-(4-hydroxybenzyl)-3-oxo-3,7-dihydroimidazo[1,2-a]pyrazin-6-yl)phenoxy)ethyl)-2-((9-(diethylamino)-5-oxo-5H-benzo[a]phenoxazin-2-yl)oxy)acetamide (5) (6-Nile-Red-CTZ)



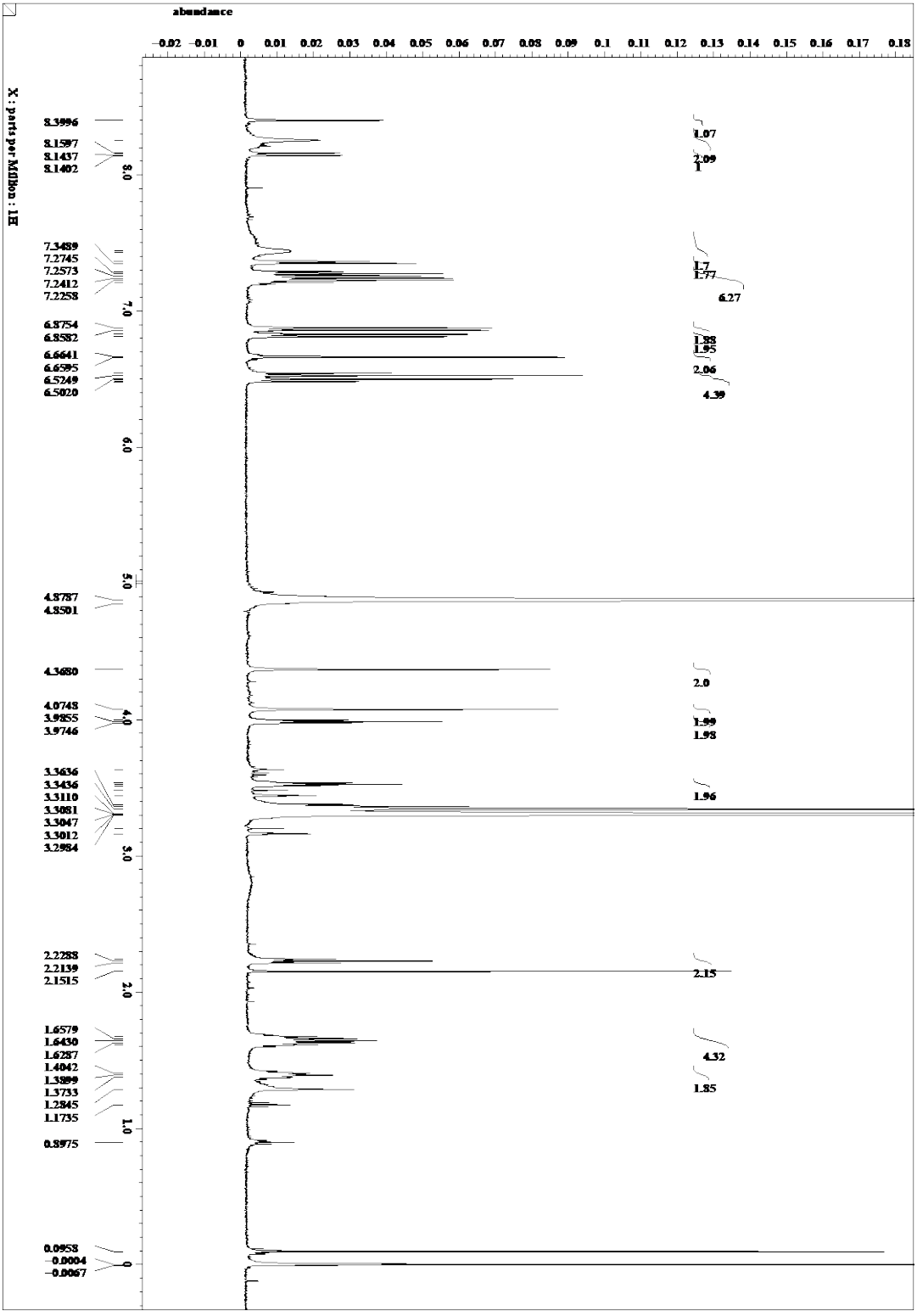
6-Chlorin-2-DMT-CTZ (6)



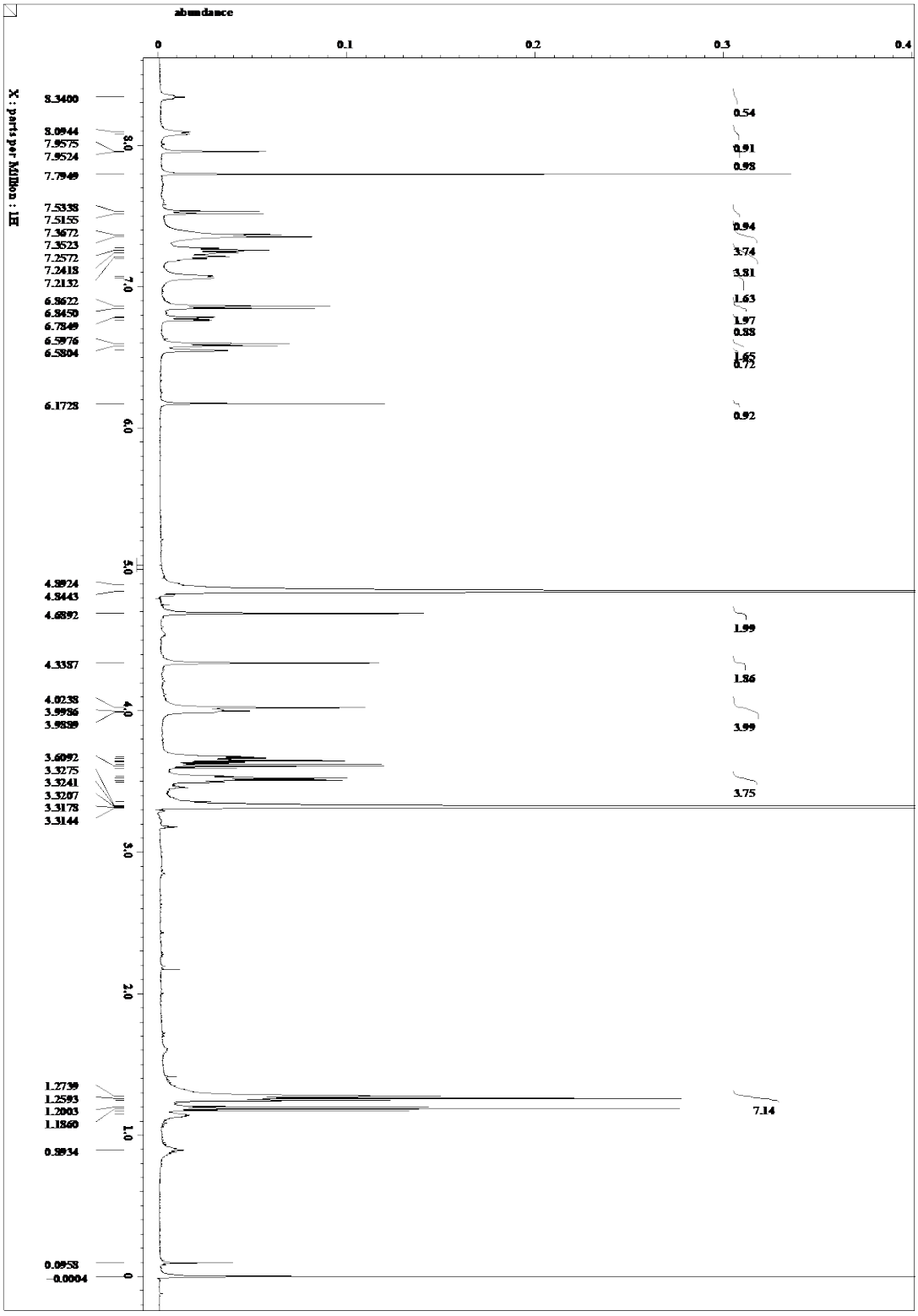
5-(3-(2-(4-(8-Benzyl-6-(4-hydroxyphenyl)-3-oxo-3,7-dihydroimidazo[1,2-a]pyrazin-6-yl)methyl)phenoxy)ethyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (8) (2-FITC-CTZ)



5-((6-((2-(4-((8-Benzyl-6-(4-hydroxyphenyl)-3-oxo-3,7-dihydroimidazo[1,2-a]pyrazin-2-yl)methyl)phenoxy)ethyl)amino)-6-oxohexyl)carbamoyl)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid (9) (2-SFX-CTZ)



***N*-(2-(4-((8-benzyl-6-(4-hydroxyphenyl)-3-oxo-3,7-dihydroimidazo[1,2-a]pyrazin-2-yl)methyl)phenoxy)ethyl)-2-((9-(diethylamino)-5-oxo-5H-benzo[a]phenoxazin-2-yl)oxy)acetamide (10) (2-NileR-CTZ)**



References

(1) Kim, S. B., Torimura, M., and Tao, H. (2013) Creation of artificial luciferases for bioassays. *Bioconjugate Chem. 24*, 2067-2075.

(2) Kim, S. B., and Izumi, H. (2014) Functional artificial luciferases as an optical readout for bioassays. *Biochem. Biophys. Res. Comm. 448*, 418-423.

(3) Inouye, S., Iimori, R., Sahara, Y., Hisada, S., and Hosoya, T. (2010) Application of new semisynthetic aequorins with long half-decay time of luminescence to G-protein-coupled receptor assay. *Anal Biochem 407*, 247-252.

(4) Lindberg, E., Mizukami, S., Ibata, K., Fukano, T., Miyawaki, A., and Kikuchi, K. (2013) Development of cell-impermeable coelenterazine derivatives. *Chem Sci 4*, 4395-4400.