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***Electronic Supplementary Information***

# A targeted delivery strategy for the development of potent trypanocides

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## A. Biological Assays

Trypanosome Growth and Viability Assays:

BSF 90-13 *T. brucei*, a 427 strain, was grown as described previously.[1,2] To score viability, parasites (1 x $10^4$/mL BSF) were seeded into 96-well clear-bottomed polystyrene plates in 200 $\mu$l HMI-9 supplemented with 10% fetal bovine serum and 10% Serum Plus (Sigma-Aldrich, St. Louis, MO) and grown in the presence of compound (2 $\mu$l) or equivalently diluted carrier for 3 days in 5% $CO_2$ at 37$^o$C. All assays were performed in triplicate. After incubation (72 hr, at 37 °C), CellTiter-Blue (Promega) was added and fluorescence measured ($560_{Ex}/590_{Em}$).

Purification of Bacterially Expressed TbHK1 and enzyme assay:

For purification of bacterially expressed TbHK1 (rTbHK1), a starter culture of *E. coli* M15(pREP) harboring pQE30 (Qiagen, Valencia, CA) with the TbHK1 gene cloned in frame of a 6-His tagging sequence was grown to an OD of ~1 and then induced for 24 hr at room temperature with 0.25 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified as described previously.[3]

HK assays were performed in triplicate as described using a coupled reaction to measure enzyme activity.[3,4] In short, the coupled assay uses glucose-6-phosphate dehydrogenase (G6PDH) to convert glucose-6-phosphate (G6-P) generated by HK to 6-phosphogluconate with coincident reduction of NADP to NADPH, which is monitored spectrophotometrically at 340 nm. Note that EbSe was found to be ineffective in a counterscreen for inhibition of G6PDH. Kinetic analyses were performed using KaleidaGraph 4.1 (Synergy Software, Reading, PA).

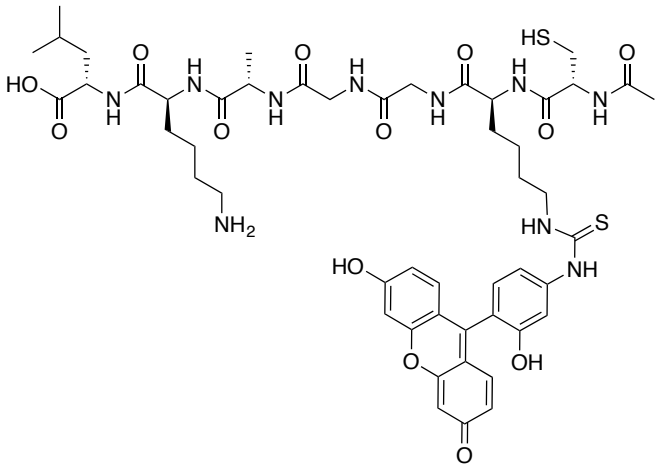
Live Cell imaging:

Bloodstream form (BSF) trypanosomes (427 strain 9013) harboring pXS6:PTSmCherry were incubated with **13** (20 μM) for 10 min before they were harvested by centrifugation at 800 g for 10 min. Cells were washed once in 1×PBS, collected by centrifugation, and resuspended in 1×PBS for live cell microscopy. Images were generated using an Axiovert 200 M microscope using Axiovision software version 4.6.3 for image analysis with **13** and the mCherry fluorescence visualized in the FITC and Texas Red channels, respectively.

Biological Data for PTS-FITC (*i.e.* lacking BABA drug) and Pentamadine Controls:

Data for Acetyl-CK(FITC)GGAKL was included to demonstrate that the PTS1 tripeptide targeting sequence itself was non-toxic to trypanosomes. Acetyl-CK(FITC)GGAKL[5] replaces the BABA drug conjugate with a flourescein isothiocyanate fluorophore. This compound effectively localizes into the

**PTS control peptide lacking BABA Drug conjugate:**

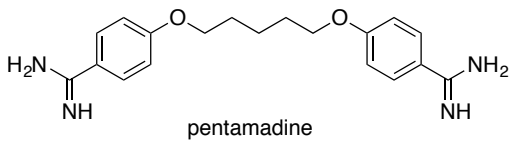


Acetyl-CK(FITC)GGAKL (See: *Biochemistry*, **2013**, *52*, 3629)

% TbHK1 inhibiton (10 μM) = 0%
$IC_{50}$ (TbHK1 inhibition) > 10 μM
% BSF *T. brucei* growth inhibition (10 μM) = 0%
$EC_{50}$ (BSF *T. brucei* lethality) >10 μM

Compound localizes into the glycosomes of BSF parasites (See: *Biochemistry*, **2013**, *52*, 3629)

**Positive control with clinically relevant pentamadine:**



pentamadine

BSF $EC_{50}$ = 0.003 ± 0.001 μM

**Scheme S1.** Biological data for PTS control peptide lacking BABA drug conjugate and positive control with clinically relevant pentamadine.

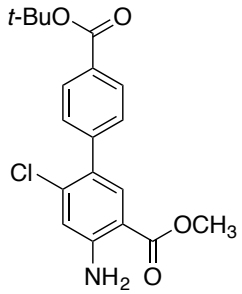
glycosomes of BSF parasites[5] but exhibits no observable toxicity against parasites nor does it inhibit TbHK1 *in vitro* (Figure S1).

Additionally, the clinically relevant anti-trypanosomal, pentamadine (Figure S1) was evaluated against BSF trypanosomes as a positive control. In our hands, pentamadine exhibited and $EC_{50}$ of 0.003 ± 0.001 μM against BSF parasites, consistent with results reported by others for the two human-infective subspecies of *T. brucei*.[6]

**B. Synthesis of BABA derivatives**

All reagents were purchased from commercial suppliers and used without further purification. Potassium carbonate was ground and dried in an oven overnight at 140 ˚C. All isolated products were purified via flash column chromatography using silica gel SDS 60 C.C. 40-63 $\mu$m. $^1$H and $^{13}$C NMR spectra were collected at ambient temperature on a 300 (Bruker Avance) or 500 MHz NMR spectrometer (Bruker Avance or JEOL Eclipse). Chemical shifts are reported in parts per million (ppm) and referenced to residual solvent peaks (i.e., $\delta$ 7.28 ppm for $^1$H NMR, 77 ppm for $^{13}$C NMR in $CDCl_3$; 2.54 ppm for $^1$H NMR, 40.45 ppm for $^{13}$C NMR in DMSO-$d_6$). Data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), and integration. Infrared (IR) spectra, reported in $cm^{-1}$, were collected using a Fourier transform spectrophotometer. Compounds **3**[7] and **9**[8] were prepared using known methods and exhibited spectral data consistent with previously reported syntheses.

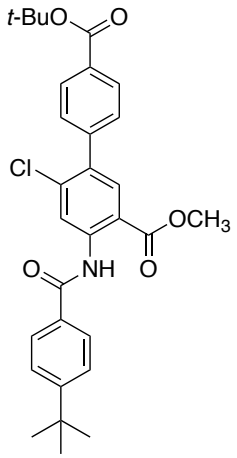
Synthesis of **10**




To a dry 100 mL round-bottomed flask was added **9** (250 mg, 0.802 mmol, 1 eq), *tert*-butylcarbonylbenzeneboronic acid (214 mg, 0.962 mmol, 1.2 eq), $PdCl_2$(dtbpf) (10.5 mg, 0.016 mmol, 0.02 eq), and potassium carbonate (168 mg, 1.203 mmol, 1.5 eq). The solids were then taken up in 9.5 mL acetonitrile and 0.5 mL water. The resulting solution was then refluxed for 48 hours under $N_2$. The reaction mixture was cooled to room temperature and filtered through a celite plug. The celite plug was rinsed with 100 mL ethyl acetate, 100 mL dichloromethane, and 100 mL methanol. The filtrate was dried with sodium sulfate, filtered, and evaporated onto a silica gel plug doped with five pipet drops of triethylamine. Product **10** was obtained as a waxy solid in 96% yield (262 mg) after silica gel column chromatography utilizing 1% triethylamine in DCM as eluent.

$^1$H-NMR (500 MHz, $CDCl_3$): $\delta$ 8.05-8.03 (d, 2H, *J* 10 Hz), 7.87 (s, 1H), 7.49-7.47 (d, 2H, *J* 10 Hz), 6.83 (s, 1H), 5.88 (s, 2H), 3.89 (s, 3H), 1.64-1.63 (s, 9H); $^{13}$C-NMR (125 MHz, $CDCl_3$): $\delta$ 167.9, 165.7, 150.4, 142.9, 138.2, 133.9, 130.8, 129.6, 129.2, 127.9, 117.3, 109.8, 81.1, 51.9, 28.3; IR ($cm^{-1}$; NaCl salt plate): 3473, 3364, 2977, 1698, 1616, 1580, 1548, 1477, 1437, 1394, 1368, 1297, 1252, 1232, 1165, 1119, 1100, 1019, 926, 848, 777, 761, 713; $R_f$ (1% $NEt_3$ in DCM): 0.633; HRMS: Calc. ($C_{19}H_{21}ClNO_4$; M+H): 362.1159, Observed: 362.1179.

Synthesis of **11**

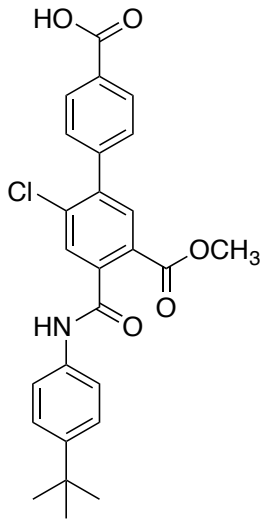



To a dry, 100 mL round-bottomed flask was added 4-*tert*-butyl benzoic acid (193 mg, 1.084 mmol, 1.1 eq) and 5 mL anhydrous DCM. Triethylamine (0.227 mL, 1.626 mmol, 1.65 eq) was added and the solution was cooled to 0 ˚C on an ice water bath. Then thionyl chloride (1 M in DCM) (1.2 mL, 1.192 mmol, 1.21 eq) was added to the solution. The solution was stirred at 0 ˚C for 30 min., then the solvent was evaporated. The residue was re-dissolved in 5 mL anhydrous dichloromethane and triethylamine (0.227 mL, 1.626 mmol, 1.65 eq) at 0 ˚C. To the acid chloride was then slowly added **10** (356 mg, 0.986 mmol, 1 eq) dissolved in 5 mL anhydrous DCM and triethylamine (0.227 mL, 1.626 mmol, 1.65 eq). The reaction mixture was stirred at 0 ˚C for 30 min, then the solution was refluxed under nitrogen overnight. The reaction mixture was cooled to room temperature and evaporated. The residue was taken up in 50 mL ethyl acetate, then washed with saturated sodium bicarbonate (3 x 50 mL) and saturated brine (50 mL). The aq. layer was washed with ethyl acetate (50 mL). The combined organics were dried over sodium sulfate, filtered, and evaporated onto silica. Product **11** was obtained

in 54% yield (338 mg) via column chromatography with 0-75% ethyl acetate in hexanes doped with 1% triethylamine.

$^{1}$H-NMR (500 MHz, $CDCl_3$): δ 12.05 (s, 1H), 9.2 (s, 1H), 8.09 (s, 1H), 8.07-8.06 (d, 2H, *J* 5 Hz), 8.01-7.99 (d, 2H, *J* 10 Hz), 7.58-7.56 (d, 2H, *J* 10 Hz), 7.52-7.50 (d, 2H, *J* 10 Hz), 3.97 (s, 3H), 1.63-1.62 (s, 9H), 1.38 (s, 9H); $^{13}$C-NMR (125 MHz, $CDCl_3$): δ 168.4, 165.7, 165.5, 156.0, 142.3, 141.9, 139.2, 133.9, 133.2, 131.5, 131.4, 129.5, 129.4, 127.4, 125.9, 121.6, 113.8, 81.3, 52.8, 35.1, 31.2, 28.3; IR ($cm^{-1}$; NaCl salt plate): 3299, 3261, 2966, 2931, 1711, 1686, 1656, 1638, 1608, 1580, 1545, 1516, 1492, 1459, 1438, 1409, 1392, 1367, 1294, 1269, 1234, 1166, 1117, 1019, 932, 899, 849, 790, 777, 761, 699; melting point = 140–142 ˚C; $R_f$ (1:1 EtOAc:1% $Et_3N$ in Hexanes) = 0.83; HRMS: ($C_{30}H_{33}ClNO_5$; M+H): Calc. 522.2047, Observed: 522.2051.

Synthesis of **12**



To a dry 100 mL round-bottomed flask was added **11** (412mg, 0.789 mmol, 1 eq) and 8.0 mL anhydrous DCM. Trifluoroacetic acid (2.6 mL, 37.5 mmol, 44.1 equiv) was then added. The reaction mixture was stirred at room temperature overnight under $N_2$. The solvent was evaporated the following day and ice-cold diethyl ether (10 mL) was added to the mixture followed by slow evaporation on the rotary evaporator. This procedure was repeated twice more. The residue was dissolved in 25 mL ethyl acetate and washed with 25 mL 1 M HCl and 25 mL saturated sodium chloride. The combined aqueous layer was washed with ethyl acetate (2 x 25 mL). The combined organics were dried with sodium sulfate, filtered, and evaporated onto silica. Product **12** was obtained in 97% yield (356 mg) after column chromatography with 1 to 50% EtOAc in hexanes followed by 3% acetic acid in hexanes/EtOAc (1:1).

$^1$H-NMR (500 MHz, DMSO-$d_6$): $\delta$ 12.99 (s, 1H), 11.71 (s, 1H), 8.87 (s, 1H), 8.04-8.02 (d, 2H, *J* 10 Hz), 7.93 (s, 1H), 7.87-7.85 (d, 2H, *J* 10 Hz), 7.61-7.59 (d, 2H), 7.55-7.54 (d, 2H), 3.89 (s, 3H), 1.312 (s, 9H); $^{13}$C-NMR (125 MHz, DMSO-$d_6$): $\delta$ 167.7, 167.4, 165.3, 156.1, 142.0, 141.3, 137.5, 133.7, 133.4, 131.4, 130.8, 129.9, 129.8, 127.4, 126.4, 121.4, 115.8, 53.4, 35.3, 31.3; IR ($cm^{-1}$; NaCl salt plate): 2958, 2923, 2853, 1732, 1685, 1653, 1636, 1608, 1579, 1559, 1541, 1519, 1507, 1491, 1457, 1437, 1419, 1377, 1312, 1268, 1233; melting point = decomposed above 260 ˚C; $R_f$ (3% AcOH in EtOAc:Hex (1:1)) = 0.43; HRMS ($C_{26}H_{25}ClNO_5$, M+H) Calc. 466.1421, Observed: 466.1248.

## C. PTS1 drug conjugate synthesis

General procedure for solid phase peptide synthesis of **4–7,** and **13** (See **Fig. S2** for structures.)

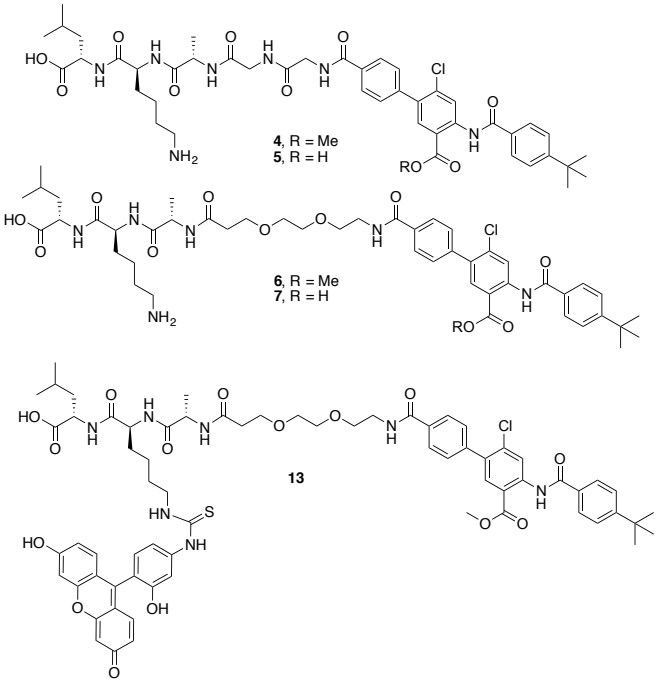



**Figure S2**: *BABA* conjugates **4**-**7**, **13.**

Peptides (in Figure S1) were assembled according to the generalized schemes presented below (i.e. Figures S3 and S4).

Resin Swelling: Fmoc-Leu-Wang resin was loaded into the barrel of a fritted 10 mL syringe and was swelled by first washing with 3 mL DCM, followed by soaking in another 5 mL DCM for five minutes. The residual DCM was washed away with DMF (3 x 5 mL).

Fmoc Deprotection Sequences: The Fmoc protecting group of the terminal amino acid of the growing peptide was removed by three sequential one-minute treatments with 5 mL of a 30% solution of 4-methylpiperidine in DMF. The deprotected resin was then washed three times with 5 mL of DMF.

HCTU amino acid couplings: Five equiv (relative to resin loading) of the appropriate Fmoc-amino acid was added to a three-dram vial, along with five equiv of HCTU. The solids were dissolved in 1 mL DMF and 5 equiv of diisopropylethylamine were added. This mixture was then taken up into the syringe and agitated for two minutes. The first treatment of the coupling mixture was then ejected from the syringe, and the resin was subsequently treated with a second aliquot of the same amino acid coupling mixture for another two minutes. After the double coupling protocol, the resin was washed with DMF (3 X 5 mL). After this coupling procedure was complete, the newly added Fmoc-amino acid was deprotected and prepared for further coupling steps as described above.

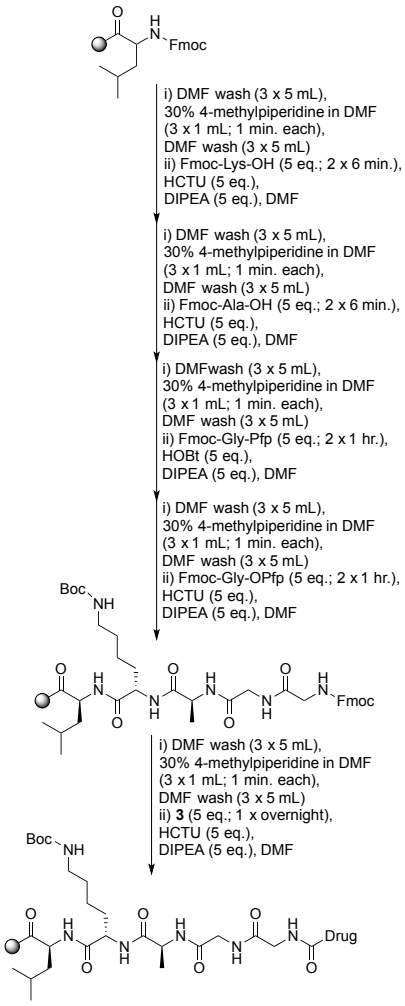



**Figure S3.** Synthesis of PTS1-drug conjugates with diglycine linker.

Fmoc-Gly-OPfp coupling (for peptides **4**, **5**, **13**): The required glycine coupling using the more reactive Fmoc-Gly-OPfp (see Figs. S3 and S4 above) was achieved in the following manner: five equiv of Fmoc-Gly-OPfp and five equiv of HOBt were charged to

two sets of three-dram vials and taken up in 1 mL of DMF. This mixture was taken up into the syringe containing the resin and was agitated for one hour. The first treatment of the coupling mixture was then ejected from the syringe, and the resin was subsequently treated with a second aliquot of the same amino acid coupling mixture for another one hour. After the double coupling protocol, the resin was washed with DMF (3 X 5 mL). After this coupling procedure was complete, the newly added Fmoc-Gly residue was deprotected and prepared for further coupling steps as described above.

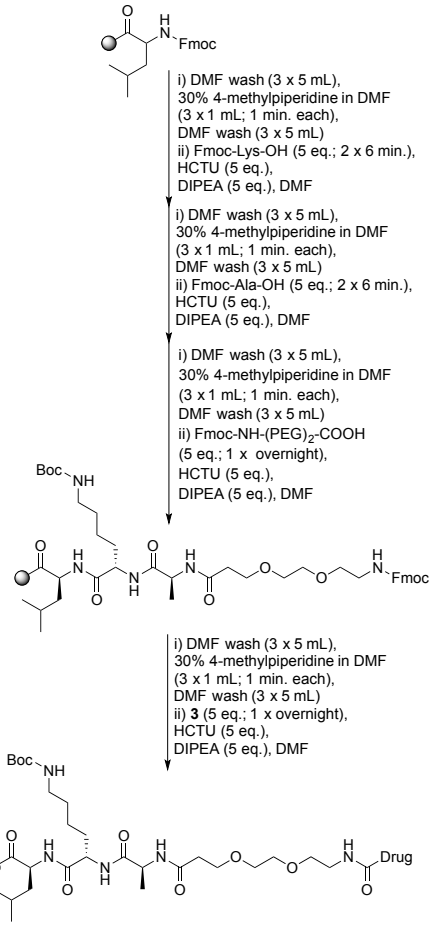



**Figure S4.** Synthesis of diethylene glycol linked PTS1-BABA conjugates.

Drug Installation: Each peptide was capped with the appropriate BABA drug candidate in the following manner: five equiv (relative to resin loading) of the appropriate BABA carboxylic acid was added to a three-dram vial, along with five equiv of HCTU. This mixture was taken up into the syringe containing the resin and was agitated overnight. After the coupling

protocol, the coupling solution was ejected from the syringe and the resin was further processed as described below.

Peptide Cleavage and Isolation: The resin containing the fully assembled peptide-drug conjugate was washed with DMF (3 x 5 mL), DCM (3 x 5 mL), and methanol (3 x 5 mL). Residual solvents were dried using suction and then the resin was further dried under vacuum (*i.e.* in a 25 ˚C vacuum oven) overnight. After drying, the resin was treated with 1.5 mL of a standard peptide cleavage cocktail comprised of trifluoroacetic acid:$H_2O$:methanol (95:4.5:0.5) for 1.5 h. The resulting solution containing the liberated peptide was then ejected into a 1 mL Eppendorf tube and evaporated under a steady stream of nitrogen. The peptide was then precipitated from the resulting oil upon the addition of ice-cold diethyl ether. The precipitate was then localized at the bottom of the tube by centrifugation, the ether portion was decanted, and the precipitation procedure was repeated two more times to afford the desired peptide-drug conjugates. The identity of the peptide-drug conjugates were then confirmed by MALDI-TOF mass spectrometry (See Table S1 below) and $^1$H NMR. Short peptides (i.e. < 8 residues) prepared using this method are typically isolated in greater than 95% purity as crude isolates.[9]

**Table S1**: MALDI-TOF data for *BABA*-PTS1 conjugates **4**-**7** and **13**.

| Conjugate | Molecular Formula | Calc. m/z | Obs. m/z |
|---|---|---|---|
| **4** | $C_{45}H_{58}ClN_7O_{10}$ | 892.4 | 892.5 |
| **5** | $C_{44}H_{56}ClN_7O_{10}$ | 878.4 | 878.5 |
| **6** | $C_{48}H_{65}ClN_6O_{11}$ | 937.5 | 937.5 |
| **7** | $C_{47}H_{63}ClN_6O_{11}$ | 923.5 | 924.2 |
| **13** | $C_{69}H_{76}ClN_7O_{16}S$ | 1326.9 | 1326.6 |

$^1$H NMR Data for Peptide-BABA conjugates:

**4:** $^1$H-NMR (500 MHz; DMSO-$d_6$): δ 11.69 (s, 1H), 8.89 (s, 1H), 8.03-8.01 (m, 3H), 7.94-7.93 (d, 2H), 7.68-7.66 (d, 2H), 7.62-7.61 (d, 2H), 3.95-3.94 (d, 2H), 3.92 (s, 3H), 3.76-3.75 (d, 2H), 1.72-1.47 (m, 8H), 1.24-1.23 (d, 3H), 0.902-0.89 (d, 3H), 0.84-0.83 (d, 3H).

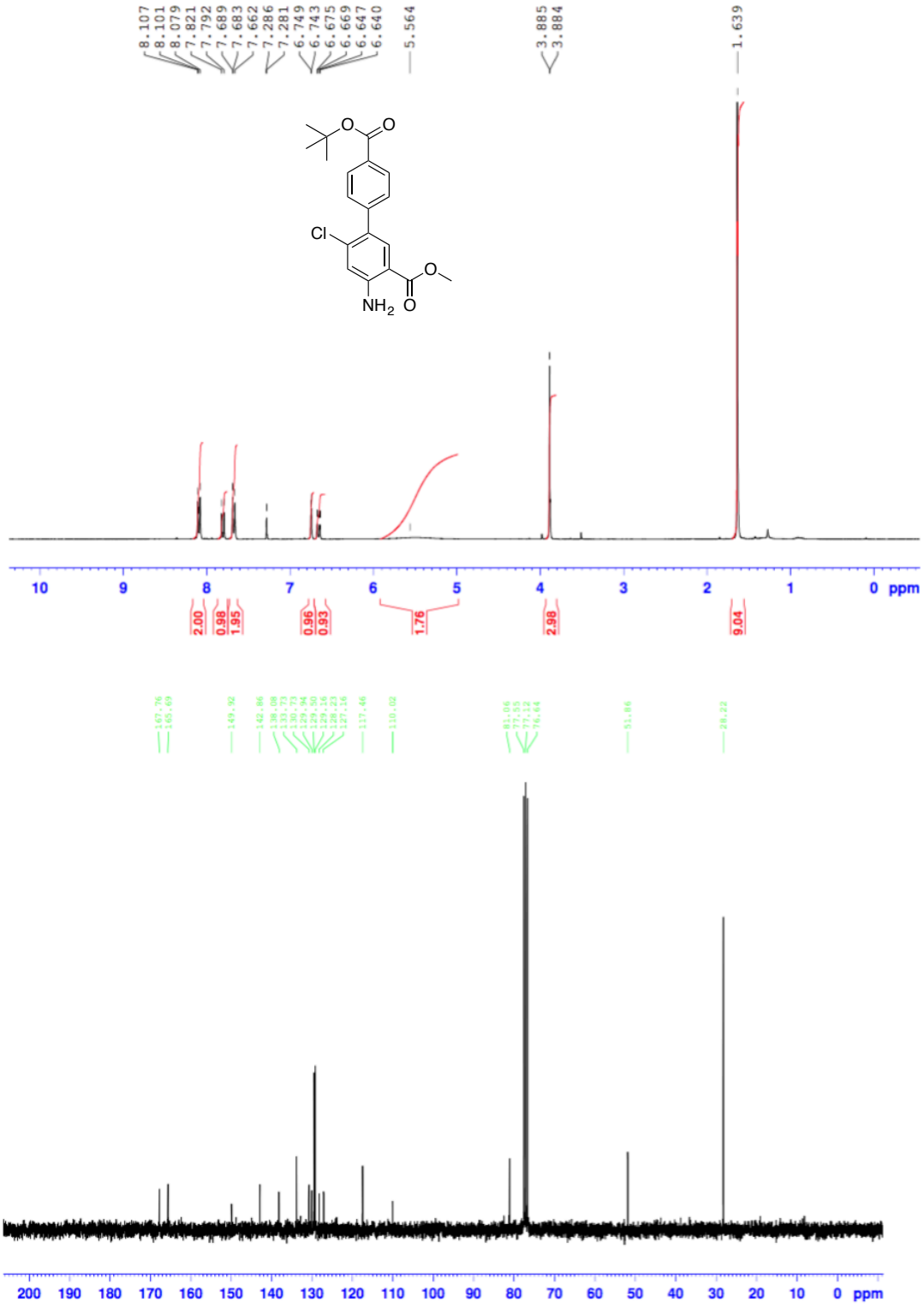
**5:** $^1$H-NMR (500 MHz; DMSO-$d_6$): 15.08 (s, 1H), 8.91 (s, 1H), 8.06 (s, 1H), 7.99-7.94 (m, 4H), 7.63-7.55 (m, 4H), 4.28-4.21 (m, 4H), 1.68-1.38 (m, 8H), 1.19-1.16 (m, 3H), 0.88-0.79 (m, 6H).

**6:** $^1$H-NMR (500 MHz; DMSO-$d_6$): δ 11.70 (s, 1H), 8.89 (s, 1H), 8.02 (s, 1H), 7.98-7.93 (m, 4H), 7.68-7.66 (d, 2H), 7.59-7.58 (d, 2H), 3.92 (s, 3H), 3.62-3.44 (m, 12H), 1.69-1.47 (m, 8H), 1.19-1.17 (d, 3H), 0.89-0.88 (d, 3H), 0.84-0.82 (d, 3H).

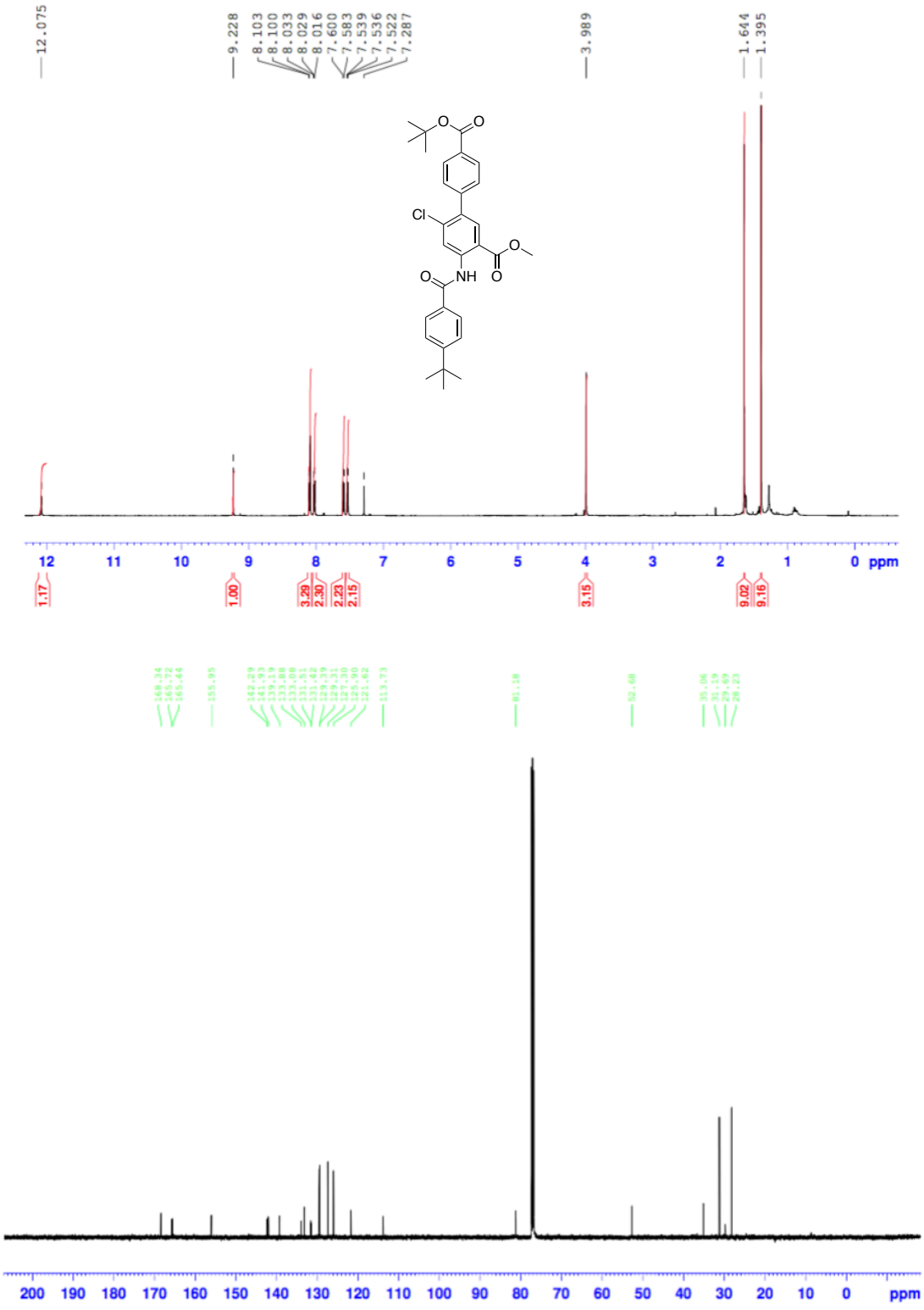
**7:** $^1$H-NMR (500 MHz; DMSO-$d_6$): δ 11.31 (s, 1H), 8.94-8.93 (s, 1H), 8.07-8.05 (m, 1H), 7.97-7.94 (m, 4H), 7.62-7.59 (m, 2H), 7.56-7.54 (d, 2H), 1.35 (m, 8H), 3.57-3.44 (m, 12H), 1.72-1.45 (m, 8H), 1.19-1.16 (m, 2H), 0.89-0.82 (m, 6H).

## D. $^{1}$H and $^{13}$C Spectra

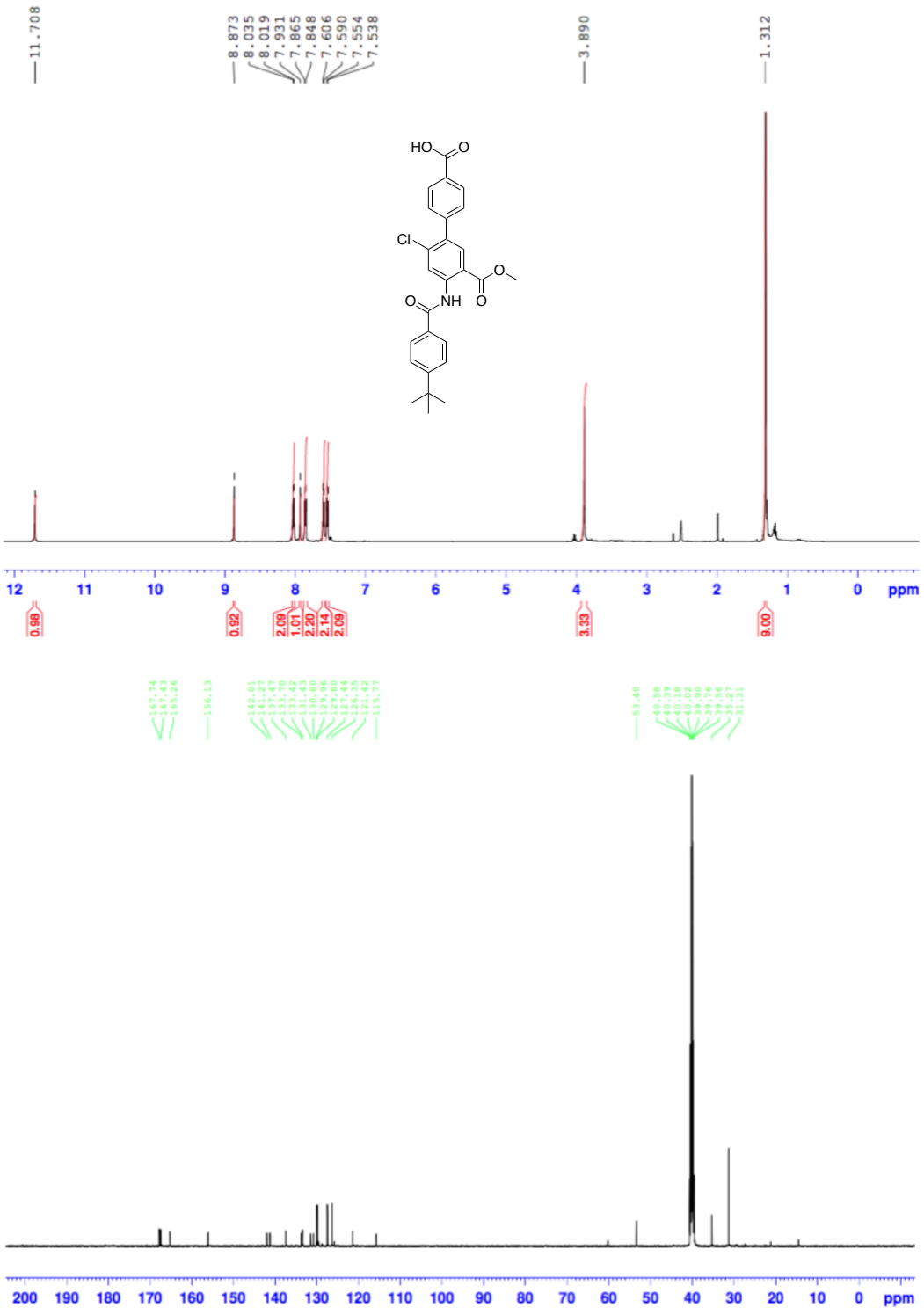
2-amino-4-chloro-5-[4-*tert*-butyloxy-carbonyl-phenyl]-methyl benzoate, **10**



2-[[4-(1,1-dimethylethyl)benzoyl]amino]-4-chloro-5-[4-*tert*-butyloxy-carbonyl-phenyl]-methyl benzoate, **11**



2-[[4-(1,1-dimethylethyl)benzoyl]amino]-4-chloro-5-[4-*tert*-butyloxy-carbonyl-phenyl]-carboxylic acid, **12**



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