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Design, synthesis, and biological evaluation of benzodiazepine-based SUMO-specific protease 1 inhibitors

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ABSTRACT

As the best-characterized ubiquitin-like protein (UBL), small ubiquitin-related modifier (SUMO) was found to conjugate with a number of proteins to regulate cellular functions including transcription, signal transduction, and cell cycle. While E1, E2 and E3 ligases are responsible for the forward SUMOylation reaction, SUMO-specific proteases (SENPs) reversibly remove SUMO from the SUMOylated proteins. Recently, SENP1 was found to be a potential therapeutic target for the treatment of prostate cancers, but the design and synthesis of its inhibitors have not been reported. We designed and synthesized a series of benzodiazepine-based SENP1 inhibitors, and they showed inhibitory activity as good as IC_{50} = 9.2 μM (compound **38**). The structure–activity relationship was also discussed.

Ubiquitin-like proteins (UBLs), such as SUMO (small ubiquitin-related modifier), NEDD8, and ISG15, were discovered to play major roles in a variety of cellular functions in eukaryotic life.[1] As the best-characterized UBL, SUMO conjugates with proteins including androgen receptor, IκBα, c-Jun, and histone deacetylases, and regulate cellular activities such as transcription, DNA repair, signal transduction, and cell cycle.[2,3] SUMOylation is a highly dynamic process: while the SUMO conjugation process is mediated by E1, E2, and E3 ligases, the reverse reaction is catalyzed by SUMO-specific proteases (SENPs).[4] Recently, SENP1 (SUMO-specific protease 1) has been found to play a critical role in prostate tumorigenesis.[5–7] It is overexpressed in more than 50% of the high-grade precancerous prostate tissues and in a large number of prostate cancer cases. Furthermore, correlation between SENP1 and increased prostate epithelial cell proliferation and precancerous structure formation was demonstrated in transgenic mice. Although the ubiquitin pathway has been explored for new cancer therapeutics and inhibitors of ubiquitin-specific proteases (USPs) have been reported,[9–11] the design and synthesis of SUMO-specific protease inhibitors have not been reported to date.[8] We believe the discovery of SENP inhibitors will open a new avenue for the search of novel cancer therapeutics.

In general, peptidyl protease inhibitors have poor pharmacokinetic properties. In peptidomimetic chemistry, incorporation of nonpeptidic scaffolds gave the advantage of improved stability and bioavailability.[13,14] As a privileged scaffold, benzodiazepine has been successfully applied as peptidomimetics: for example, somatostatin mimetics, caspase-3 inhibitors, and falcipain-2 inhibitors.[13,15,16] We report herein the design, synthesis, and biological evaluation of benzodiazepine-based SENP1 inhibitors. It is the first time the design and synthesis of SENP1 inhibitors were reported.

SENP1 processes the precursor SUMO to its mature form by catalyzing the cleavage of a scissile peptide bond. In the crystal structure of human SENP1 complexed with unprocessed SUMO1 (PDB: 2IY1),[12] the catalytic Cys603 is located in a cleft which, upon substrate binding, closes to form a channel-like structure. The terminal peptidic tail of the precursor SUMO1 occupies the cleft with the formation of a number of hydrogen bonds. The benzodiazepine core structure **8** was docked into the above catalytic cleft and was found to be able to mimick the conformation of the substrate peptide with the formyl group forming a covalent bond with Cys603. Furthermore, substitution at the C(3)–NH_2 may take an orientation analogous to the substrate peptide chain. Thus, a series of benzodiazepine derivatives as depicted in Scheme 1 were synthesized based on scaffold **8**.

The synthesis of intermediate **3** was adapted from Sherrill et al.[17] with improved yields (Scheme 1). First, the three-component condensation gave α-benzotriazolylglycine derivative **1** in 71% yield. Coupling with 2-amino benzophenone under the condition of EDC/DMAP gave amide **2** in quantitative yield. Subsequent

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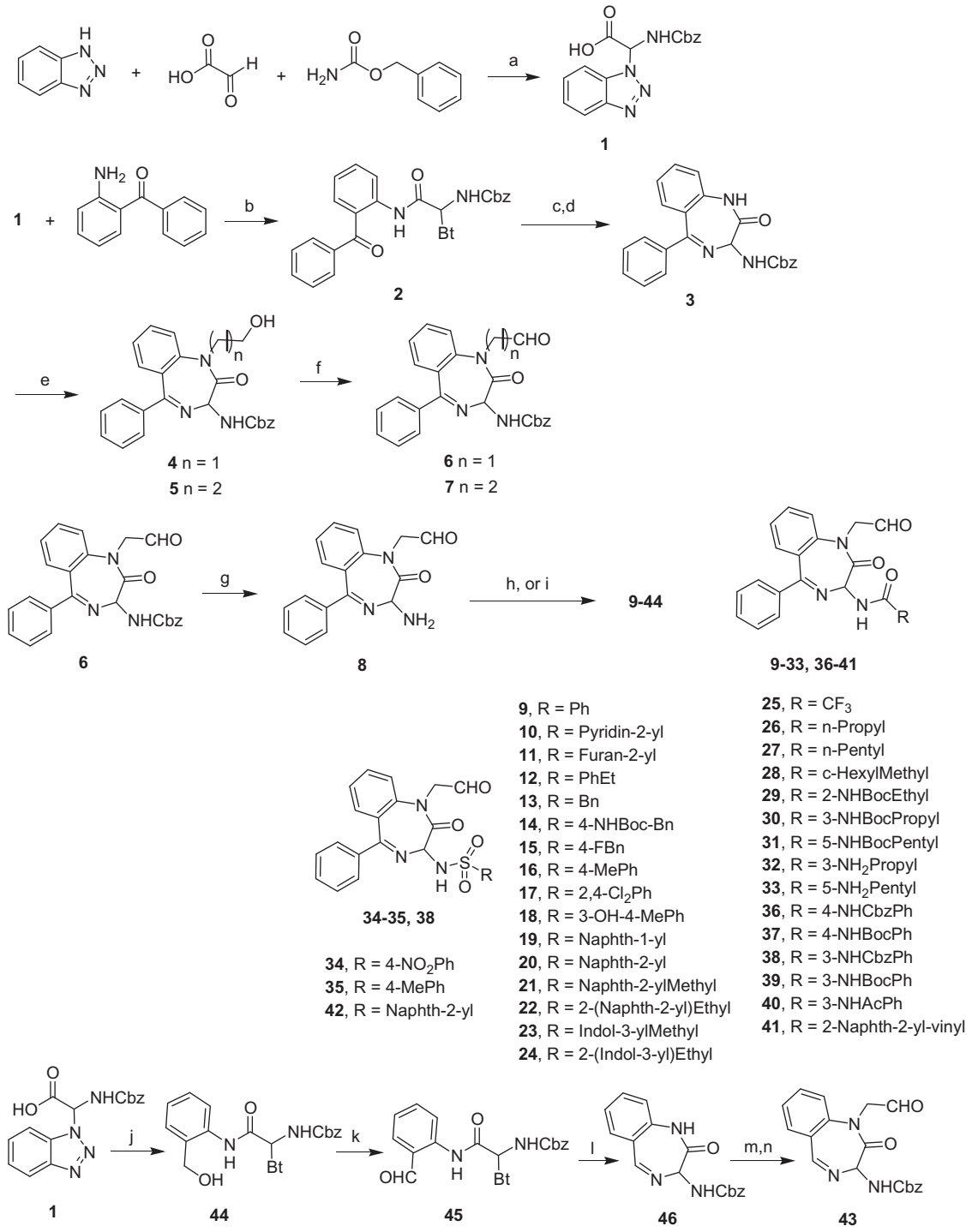

Scheme 1. Reagents and conditions for compounds **9**–**44**: (a) toluene, rt 30 min, then reflux 2 h (71%); (b) 1.5 equiv EDC, cat. DMAP, CH_2Cl_2, 0 °C to rt (99%); (c) 2 M NH_3 in EtOH, rt, 3 h; (d) NH_4Oac, AcOH, rt, overnight (71%); (e) 1.3 equiv 2-hydroxyethyl tosylate or 3-hydroxypropyl tosylate, K_2CO_3, MeCN, reflux, 20-40 h (67%-**4**, 83%-**5**); (f) PCC, 4 Å MS, CH_2Cl_2, rt, overnight (71%-**6**, 44%-**7**); (g) 10% Pd/C, 1 atm H_2, rt, overnight (quant.); (h) 1.5 equiv RCOOH, cat. DMAP, 1.5 equiv EDC, CH_2Cl_2, 0 °C to rt (9–54%); (i) 1.5 equiv RCOCl or 2 equiv RSO_2Cl, NEt_3, CH_2Cl_2, 0 °C to rt, overnight (17–40%); (j) 1 equiv 2-aminophenylmethanol, DCC, NHS, CH_2Cl_2, 0 °C to rt, 1 h (95%); (k) see (f) (42%); (l) see (c) (67%); (m) see (e) (60%); (n) see (f) (60%).

cyclization was achieved with 71% yield over two steps, which involved treatment with ethanolic ammonia followed by ammonium acetate in acetic acid, to give benzodiazepine **3**. To introduce the aldehyde group from N(1), a number of approaches including the use of bromoacetaldehyde, 3-bromopropanal, 2-bromoethanol, and 3-bromopropanol were explored. Finally, 2-hydroxyethyl or 3-hydroxypropyl tosylate in the presence of K_2CO_3 gave alcohol **4** or **5** in 67% or 83% yield respectively. Afterwards, **6** and **7** were prepared by PCC oxidation of **4** and **5** with 71% and 44% yields, respectively. The benzodiazepine **8**[18] was subsequently obtained by catalytic hydrogenation in the presence of Pd/C with quantitative yield. Thus, intermediate **8** was successfully obtained in large scale via this robust route.

Synthesis of the amide analogs **9**–**33** and **36**–**41**, the sulfonamide analogs **34**, **35** and **42**, and the 5-H analog **43** were described in Scheme 1. The amide analogs were prepared by coupling the

amine group of **8** either with acyl chlorides in the presence of Et_3N or with carboxylic acids under the conditions of EDC/DMAP. The sulfonamide analogs were prepared from **8** and sulfonyl chlorides in a similar manner. In order to synthesize the 5-H analog **43**, glycine intermediate **1** was coupled with 2-aminophenylmethanol in the presence of DCC/NHS to give amide **44**, which was oxidized with PCC to give aldehyde **45**. Cyclization under the condition of ethanolic ammonia gave 5-H benzodiazepine **46**, which was converted to the corresponding aldehyde **43** by reaction with 2-hydroxyethyl tosylate followed by oxidation with PCC.

In order to evaluate the inhibitory effect of the synthesized benzodiazepines, SENP1 and SUMO-ΔRanGAP were cloned, expressed, and purified.[19] The fluorescence assay was carried out using SUMO-CHOP-Reporter kit (LifeSensor Inc. Malvern, PA): The SUMO–reporter–enzyme fusion protein is cleaved by SENP1 to release and activate the reporter enzyme (phospholipase A_2) which subsequently convert its substrate (2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholin) to a fluorescent form to give a quantitative measurement of the isopeptidase activity of SENP1.[20,21] At the same time, a second assay based on the cleavage of SUMO-ΔRanGAP by SENP1 and gel electrophoresis analysis was also developed.[22]

The above synthesized benzodiazepines were first screened at 20 μM against SENP1 using the fluorescence assay, and the result was summarized in Table 1. First, different aromatic carboxylic acids were used to generate carboxamides **9**–**18**. They showed no inhibition except the minimal activity of compound **14** which has a Boc-protected aminomethyl group at the C(4′) of the phenyl and represents the most spatially extended molecule in this series. It suggests that extended substituents on the phenyl may capture additional interactions with the enzyme and yield improved affinity. Sulfonamide **34** did not show any inhibitory activity, while **35** gave a minimal activity which is an improvement from its amide analog **16**. Next, fused aromatic substituents were explored as demonstrated by amides **19**–**24**. The 2-naphthyl compound **20** showed 30% inhibition, while the 1-naphthyl substituted **19** showed no inhibition. Thus, extended 2-naphthyl compounds **21** and **22** were synthesized. Compound **21** showed similar activity, while compound **22** gave enhanced inhibition of 45%, which corroborates with the observation that spatial extension of the substituents may better occupy the binding site and give improved affinity. In the case of indolyl compounds **23** and **24**, compound **24** with an ethylene linker was more active than compound **23** with a methylene linker. Furthermore, alkyl carboxamides **25**–**33** were explored as well. Alkyl analogs **25**–**28** with nonpolar aliphatic substituents were inactive. It is interesting to observe that although compounds **29**–**31** with Boc-protected amino groups were inactive, the aminoalkyl carboxamide **33** showed an inhibition of 43%. Again, the shortened analog **32** gave minimal activity.

Table 1
Percentage inhibition of SENP1 by compounds **9**–**35** at 20 μM

Compd	% Inhibition at 20 μM	Compd	% Inhibition at 20 μM
9	0	**23**	3
10	7	**24**	24
11	0	**25**	0
12	2	**26**	0
13	1	**27**	0
14	23	**28**	5
15	0	**29**	0
16	0	**30**	0
17	0	**31**	0
18	5	**32**	4
19	7	**33**	43
20	30	**34**	0
21	26	**35**	17
22	45		

Table 2
IC_{50} values[a] of compounds with percentage inhibition ⩾50% at 20 μM against SENP1, and their structurally related compounds (inhibition >50%) were also included in the table for SAR comparison

Compd	IC_{50} (μM) SENP1	Compd	IC_{50} (μM) SENP1
36	15.5	**42**	21.2
37	>100	**6**	27.2
38	9.2	**4**	>100
39	>100	**43**	>100
40	>100	**7**	>100
41	21.8		

[a] IC_{50} values were measured in duplicates using 50 μM nonspecific peptidic protease inhibitor as a positive control. A solution containing SENP1, SUMO-ΔRanGADMSO, and 0.1% DMSO was used as negative control.

Based on the above screening results, we synthesized a series of carboxamides with extended substituents on the phenyl and a number of these compounds showed inhibitory activity above 50%. Thus, IC_{50} values were measured by the fluorescence assay. Installation of NHCbz at the C(4′) of the phenyl led to significantly improved activity (**36**[23]: IC_{50} = 15.5 μM). In the case of NHBoc group (**37**), the activity was abolished. When the NHCbz group was moved to C(3′), compound **38**[24] also showed good potency (IC_{50} = 9.2 μM). When the C(3′) substituent was NHBoc or NHAc (**39** and **40**), the activity was completely abolished, which suggests that the corresponding binding area may have a propensity for aromatic moieties. Carboxamide **41** and sulfonamide **42** with fused aromatic rings showed IC_{50} of 21.8 and 21.2 μM, respectively. The lead compound **38** was also subjected to the SUMO–ΔRanGAP cleavage assay and inhibited the enzymatic hydrolysis of SUMO-ΔRanGAP by SENP1 (Fig. 1).

We next explored the roles of the formyl group, the C(5) phenyl group, and the distance between the formyl and the benzodiazepine core, using compound **6** as a model. Reduction of the formyl group (**4**) led to complete loss of potency, which corroborates with the formation of a covalent bond with Cys603. Deletion of the C(5) phenyl group (**43**) also resulted in loss of activity. Lengthening the distance between the formyl group and the 7-membered core (**7**) led to diminished activity as well.

SENP1–**38** complex was modeled based on SENP1 structure (PDB: 2IY1) (Schrodinger, Inc.) (Fig. 2). Besides the covalent bond with Cys603, the following favorable interactions were observed:

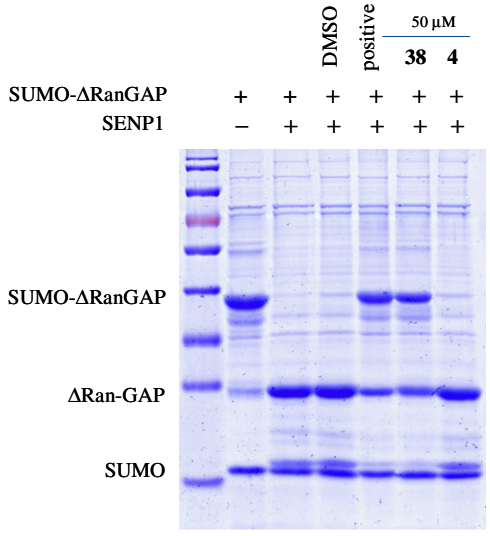

Figure 1. Cleavage of SUMO-ΔRanGAP by SENP1 was inhibited by **38** but was not affected by **4** at 50 μM. Positive and negative controls were as described in Table 2.

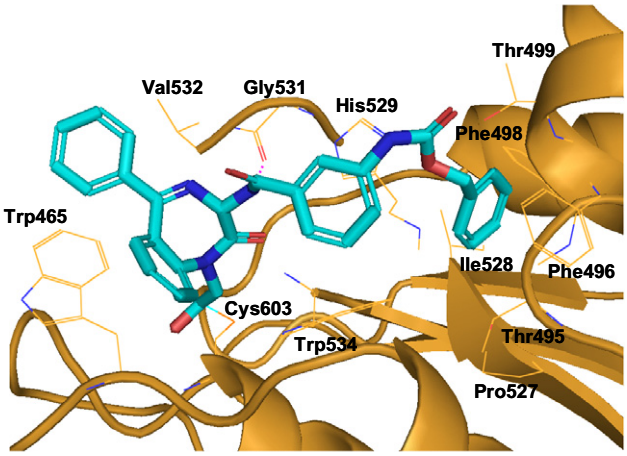

Figure 2. Binding model of **38** in SENP1 catalytic site. Cyan sticks-**38**, yellow ribbons-SENP1 protein, yellow lines-SENP1 residues, magenta dashed line-hydrogen bond.

hydrogen bond between C(3)–NH and Gly531; π–π interaction of Trp465 with C(5)- and diazepine-fused phenyls; π–π interaction of C(3)NH-phenyl and His529; nonpolar interaction between C(5)-phenyl and Val532. The phenyl group of the Cbz moiety inserts into a hydrophobic pocket consisting of residues Phe496, Thr495, Thr499, Phe498, Ile528 and Pro527. In general, these observations were corroborative with the structure–activity relationship discussed above.

The compounds with the best SENP1 inhibitory activity were tested against prostate cancer cells (PC3) to evaluate their ability to inhibit cancer cell growth in vitro. The lead SENP1 inhibitors **36** and **38** showed IC_{50} values of 13.0 and 35.7 μM against prostate cancer cells, respectively.

In summary, we designed and synthesized a series of SENP1 inhibitors based on benzodiazepine scaffold. They showed inhibitory activity as good as IC_{50} = 9.2 μM. This is the first report on design and synthesis of SENP1 inhibitors. Further study is underway to obtain inhibitors with improved potency and druggability and to eventually utilize them to validate the novel therapeutic target.

Acknowledgments

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18. *Spectroscopic data for **8***: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.67 (s, 1H), 7.65–7.34 (m, 8H), 7.17 (d, J = 8.0 Hz, 1H), 4.78–4.60 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 195.78, 170.42, 166.52, 141.70, 138.18, 131.95, 130.61, 130.37, 129.73, 129.63, 128.31, 124.92, 121.43, 70.10, 57.71; HRMS (ESI) $[M+H]^+$ $C_{17}H_{16}N_3O_2$ calcd 294.1243, found 294.1220.
19. The DNA of the catalytic domain of SENP1 (SENP1c, aa419–aa643) was amplified from PC3 cell cDNA library, cloned into pET28a(+) vector, and expressed in *Escherichia coli* BL21 by induction with 0.2 mM isopropyl β-D-thiogalactoside at 25 °C for 12 h. Cell pellets were resuspended in buffer A (300 mM NaCl, 50 mM pH 8.0 PBS, 10 mM imidazole, 0.2 mM PMSF and 2 mM β-mercaptoacetic ethanol) and lysed by sonication on ice. The his-tagged protein was purified by affinity chromatography using Ni-NTA-agarose (QIAGEN) and eluted with 250 mM imidazole in buffer A. The SUMO–ΔRanGAP plasmids were kindly provided by Dr. Jinke Cheng (SJTU School of Medicine). Expression and purification of SUMO-ΔRanGAP followed similar methods.
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21. *SUMO-CHOP-reporter fluorescence assay*: SENP1 (20 nM) was preincubated with compounds for 10 min at 37 °C before the SUMO–reporter–enzyme and the reporter substrate were added and incubated for 30 min. The fluorescence intensity was determined on the Synergy H4 Hybrid Microplate Reader plate reader with excitation and emission at 475 and 555 nm, respectively, at room temperature. The relative activity of SENP1 was determined by measuring the RFU value. Net RFUs were determined by subtracting the blank RFU value (the reporter substrate in assay buffer) from each data point. Percentage inhibition was calculated using the following equation $[1-(RFU_{compd}/RFU_{DMSO})] \times 100$. IC_{50} values were determined using a sigmoidal dose–response (variable slope) model.
22. *SUMO-ΔRanGAP cleavage assay*: After preincubation of SENP1 with compounds for 10 min at 37 °C, SUMO-ΔRanGAP was added and incubated for 30 min. The reaction was stopped by boiling in sample buffer. The proteins were separated by SDS–PAGE and visualized by coommassie blue stain for SUMO-ΔRanGAP cleavage.
23. *Spectroscopic data for **36***: ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.66 (s, 1H), 7.91–7.86 (m, 3H), 7.65-7.56 (m, 3H), 7.51–7.29 (m, 11H), 7.21 (d, J = 8.1 Hz, 1H), 6.87 (s, 1H), 5.86 (d, J = 8.1 Hz, 1H), 5.22 (s, 2H), 4.74 (s, 2H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.17, 168.08, 168.01, 166.39, 152.92, 141.29, 138.07, 135.78, 132.24, 130.89, 130.82, 129.96, 129.76, 128.65, 128.60, 128.45, 128.36, 125.47, 121.71, 117.88, 67.45, 67.25, 57.71 ppm; HRMS (ESI) $[M+1]^+$ $C_{32}H_{27}N_4O_5$ calcd 547.1981, found 547.1982.
24. *Spectroscopic data for **38***: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.66 (s, 1H), 7.93 (d, J = 8.0 Hz, 1H), 7.80–7.79 (m, 2H), 7.65–7.57 (m, 4H), 7.50–7.28 (m, 10H), 7.22 (d, J = 8.4 Hz, 1H), 6.81 (s, 1H), 5.85 (d, J = 8.0 Hz, 1H), 5.22 (s, 2H), 4.74 (s, 2H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.23, 168.09, 167.89, 166.69, 153.23, 141.27, 138.34, 138.03, 135.94, 134.67, 132.24, 130.87, 130.82, 129.91, 129.77, 129.43, 128.60, 128.31, 128.28, 125.44, 122.02, 121.72, 117.65, 67.55, 67.10, 57.70 ppm; HRMS (ESI) $[M+1]^+$ $C_{32}H_{27}N_4O_5$ calcd 547.1981, found 547.1980.