Synthesis and pharmacological characterization of dibenzodiazepinone-type muscarinic M_2-receptor antagonists conjugated to fluorescent dyes or small peptides

DISSERTATION

ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN
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vorgelegt von
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PUBLICATIONS, PRESENTATIONS AND PROFESSIONAL TRAINING

Peer- reviewed journal articles

(published or submitted prior the submission of this thesis)

A. Pegoli*, D. Wifling*, **C.G. Gruber***, X. She, H. Hubner, G. Bernhardt, P. Gmeiner, M. Keller, Conjugation of short peptides to dibenzodiazepinone-type muscarinic acetylcholine receptor ligands determines M_2R selectivity, J. Med. Chem., 62 (**2019**) 5358-5369.
*equally contributed

X. She, A. Pegoli, **C.G. Gruber**, D. Wifling, J. Carpenter, H. Hübner, J. Wan, G. Bernhardt, P. Gmeiner, N.D. Holliday, M. Keller, Red-emitting dibenzodiazepinone derivatives as fluorescent dualsteric probes for the muscarinic acetylcholine M_2 receptor, J. Med. Chem., 63 (**2020**) 4133-4154.

C.G. Gruber, A. Pegoli, C. Müller, L. Grätz, X. She, M. Keller, Differently fluorescence-labelled dibenzodiazepinone-type muscarinic acetylcholine receptor ligands with high M_2R affinity, RSC Med. Chem., 11 (**2020**) 823-832.

L. Grätz, T. Laasfeldb, A. Allikaltb, **C. G. Gruber**, A. Pegoli, M.-J. Tahk, M.-L. Tsernant, M. Keller, A. Rinken, BRET- and fluorescence anisotropy-based assays for on-line monitoring of ligand binding to muscarinic M_2 receptors, Biochim. Biophys. Acta, Mol Cell Res., **2020**, submitted

C. G. Weinhart, D. Wifling, M. F. Schmidt, E. Neu, C. Höring, T. Clark, P. Gmeiner and M. Keller., Dibenzodiazepinone-type muscarinic receptor antagonists conjugated to basic peptides: impact of the linker moiety and unnatural amino acids on M_2R selectivity, Eur. J. Med. Chem., **2020**, submitted

Poster presentations

C. Gruber, A. Pegoli, D. Wifling, G. Bernhardt, M. Keller, Conjugation of dibenzodiazepinone-type muscarinic acetylcholine receptor ligands to short peptides as a new strategy for the development of highly selective M_2R ligands, *8th Austrian Peptide Symposium, December 2018, Salzburg, Austria*

C. Gruber, A. Pegoli, D. Wifling, G. Bernhardt, M. Keller*,* Conjugation of dibenzodiazepinone-type muscarinic acetylcholine receptor ligands to short peptides as a new strategy for the development of highly selective M_2R ligands, *9th International Summer School "Medicinal Chemistry", September 2018, Regensburg, Germany*

C. Gruber, A. Pegoli, D. Wifling, M. Keller, Conjugation of dibenzodiazepinone-type muscarinic acetylcholine receptor ligands to short peptides as a new strategy for the development of highly selective M_2R ligands, *Frontiers in Medicinal Chemisty, March 2020, Freiburg, Germany (cancelled due to Corona pandemic)*

Professional training

Since January 2017 member of the research training group 1910 “*Medicinal Chemistry of Selective GPCR Ligands*” funded by the Deutsche Forschungsgemeinschaft

CHAPTER 1

General introduction

1.1. G-Protein coupled receptors

1.1.1. GPCRs: Overview and classification

G-protein coupled receptors (GPCRs), also referred to as seven transmembrane (7TM) receptors, are versatile proteins constituting the largest and most intensively studied family of membrane receptors. As they are responsible for transducing signals across plasma membranes, GPCRs are essential nodes of communication between the intra- and extracellular environment.[1, 2] Hence, GPCRs are substantially involved in the regulation of physiological processes, and represent attractive and important drug targets.[3-7] GPCRs are encoded by approximately 800 genes in humans and consist of a single polypeptide chain, which contains seven hydrophobic alpha helices spanning the plasma membrane.[8, 9] The N-terminus is located extracellularly and the C-terminus intracellularly. Consequently, GPCRs exhibit three extracellular and three intracellular loops, located between the extracellular ends and the intracellular ends, respectively, of adjacent transmembrane domains.[10, 11] In humans, about 450 GPCRs have sensory function (olfaction, taste, light perception, pheromone signaling) and ca. 350 GPCRs are non-sensory receptors, transmitting an extracellular stimulus into the cell by binding of an (endogenous) ligand, acting as an agonist.[12] GPCRs bind a tremendous variety of ligands, covering small molecules such as biogenic amines (e.g. acetylcholine), peptides (e.g. neuropeptide Y), nucleotides and Ca^{2+} ions, as well as proteins (e.g. the 28 kDA CXCR6 chemokine receptor agonist CXCL16), pheromones and exogenous ligands such as fragrances or flavours (Figure 1.1).[12, 13]

Based on sequence homologies and functional roles, the vertebrate GPCRs can be classified into five families: rhodopsin-like receptors (class A), secretin receptor-like GPCRs (class B), class C GPCRs (comprising metabotropic glutamate receptors, γ-aminobutyric acid receptors and Ca^{2+}-sensing receptors), adhesion GPCRs and the family of frizzled proteins.[12] With ca. 430 sensory and 300 non-sensory receptors, class A comprises most GPCRs.[12] Approximately 80 non-

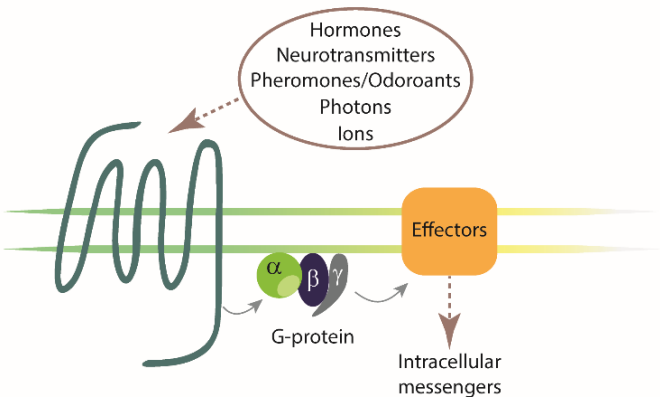


Figure 1.1. Schematic illustration of a generic GPCR and a heterotrimeric G-protein. GPCRs exhibit seven transmembrane helices (7TM) with an extracellular N-terminal and an intracellular C-terminal domain. Reception of an extracellular stimulus (e.g. photons, ions, odorants, pheromones, hormones, neurotransmitters) induces conformational changes in the receptor that mediates the activation of heterotrimeric G-proteins. G-proteins, in turn, interact with a various effectors, controlling intracellular messengers. (modified from literature[11])

sensory receptors of class A are still orphans, i.e. an endogenous ligand has not been identified.[12, 14]

As GPCRs are involved in the regulation of numerous physiological functions (e.g. smooth muscle contraction, pain modulation, cognition, cell proliferation, heart function, *etc.*), dysfunctions of GPCRs are associated with a multitude of different diseases, such as asthma, diabetes type 2, schizophrenia (SZ), Alzheimer's disease (AD) or various autoimmune diseases.[15] This, and the fact that GPCRs are easily druggable, account for their predominating role among biological targets of approved drugs, hence, to date, drugs that target GPCRs account for approx. 27% of the global market share of therapeutic drugs.[15]

1.1.2. G-protein-dependent signaling

The guanine nucleotide-binding proteins (G-Proteins) are heterotrimeric proteins, consisting of an α-subunit, exhibiting GTPase activity, a β-subunit and a γ-subunit.[16] In its inactive, GDP-bound form, the α-subunit is tightly connected to the β/γ-heterodimer. Typically, the active conformational state of a GPCR is induced by binding of an agonist to the orthosteric binging site of the receptor. Consequently, the G-protein complex associates with the intracellular site of the receptor *via* the GDP bound α-subunit, resulting in the release of GDP and the rapid binding of GTP instead. This, in turn, results in the dissociation of the GPCR/G$\alpha\beta\gamma$ complex into the GPCR, the α-subunit and the $\beta\gamma$-complex.[11, 17] Both components of the G-protein are then capable of activating different downstream effectors. In its GTP bound state, the α subunit is "switched" into its active form, which can associate with its respective downstream effector proteins, including adenylyl cyclases (ACs), phospholipase C-β or ion channels.[11, 16, 18] Effector proteins, addressed by the β/γ-subunit, are, e.g., ACs, GPCR kinases (GRKs) or ion channels.[19] Upon hydrolysis of GTP to GDP by the intrinsic GTPase activity of the α-subunit, the latter is "switched off", i.e. turns back to the inactive state resulting in the re-association of the α- and β/γ-subunits, which can then undergo a new activation cycle.[20] As various isoforms of α-, β- and γ-subunits exist, multiple heterotrimeric G-proteins can be constituted, exhibiting distinct effector interaction profiles.[11, 21, 22] Based on their sequence homology and function, Gα-subunits were subdivided into four groups (α_s, $\alpha_{i/o}$, $\alpha_{q/11}$ and $\alpha_{12/13}$).[13, 17, 20, 22] Gα_s is responsible for the stimulation of ACs, leading to an increase of intracellular cAMP (3'-5'-cylclic adenosine monopohsopate) levels and consequently to an activation of proteinkinase A (PKA),[16, 22] whereas its counterpart, G$\alpha_{i/o}$, leads to an inhibition of the enzymatic activity of ACs and thus in decreased intracellular cAMP formation (Figure 1.2).[22, 23] A third major Gα subtype

represents $G\alpha_{q/11}$, which activates effector proteins from the phospholipase C-β (PLC-β) class (Figure 1.2). PLC-β catalyses the formation of inositol-1,4,5-trisphosphat (IP_3) and 1,2-diacylclygerol (DAG) by hydrolysis of phosphatidylinositol 4,5-bisphpsophate (PIP_2).[24] IP_3 is a second messenger that mediates the efflux of Ca^{2+} from intracellular stores, in particular from the endoplasmatic reticulum (ER).[25] DAG, however, activates protein kinase C (PKC), which is responsible for the phosphorylation of various proteins.[25] The β- and γ-subunits, forming a tightly associated βγ-complex, were also identified as signal transducers. They transmit signals to various downstream effector proteins, such as ACs[26], PLCs[27] and different types of ion channels[28-30]. Furthermore, the GPCR signaling is essentially influenced by receptor expression as well as by desensitization and internalization of activated receptors. Upon agonist binding, the activated receptor is prone to phosphorylation at specific sites at the intracellular loops and carboxyl-terminal tail trough, e.g., GRKs.[31] This results in a decreased G-protein binding to the receptor, but in an increased affinity of β-arrestin, which also binds to the intracellular side of the receptor mediating its internalization.[31, 32]

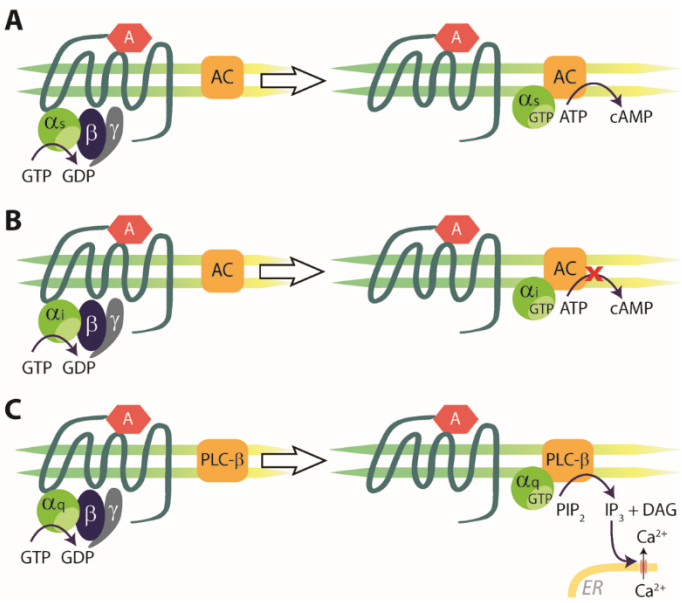


Figure 1.2. Schematic overview of major GPCR signalling pathways. Agonist ("A") binding results in an exchange of GDP by GTP, leading to a dissociation of the αβγ-complex in a α–subunit and βγ-dimer. (A) Stimulation of the $G\alpha_s$-subfamily activates ACs leading to increased cAMP formation from ATP. (B) Activation of $G\alpha_i$-coupled GPCRs leads to a decrease in AC activity. (C) Activation of $G\alpha_q$-subunits results in a stimulation of PLC-β, which then promotes the formation of IP_3 and DAG. The second messenger IP_3 mediates the release of Ca^{2+} from the ER (modified from literature[22])

1.2. Muscarinic acetylcholine receptors

1.2.1. General aspects, expression and physiological functions

In humans, the family of muscarinic acetylcholine receptors (MRs) comprises five subtypes, designated M_1R, M_2R, M_3R, M_4R and M_5R.[33] Whereas the M_1R, M_3R and M_5R preferably couple to G_q-type G-proteins, resulting in phospholipase C activation, hydrolysis of PIP_2 and an increase in intracellular Ca^{2+}, the M_2 and M_4 receptor mainly activate $G_{i/o}$-type G-proteins, leading to a reduction of cAMP formation by the inhibition of ACs.[33-35] Physiologically, MRs and nicotinic acetylcholine receptors (nAChRs) are activated by acetylcholine (ACh) (*cf.* Figure 1.3), which binds to the five MRs with rather low affinity: pK_i values: 4.9 (M_1R),[36] 4.3-

6.5 (M_2R),[37-39] 4.5-5.4 (M_3R)[36-38], 4.5-5.6 (M_4R),[36-38] 6.1 (M_5R)[37]. The neurotransmitter ACh is formed by choline acetyl transferase from acetyl-Coenzyme A and choline, and degraded by acetylcholinesterase (Figure 1.3).

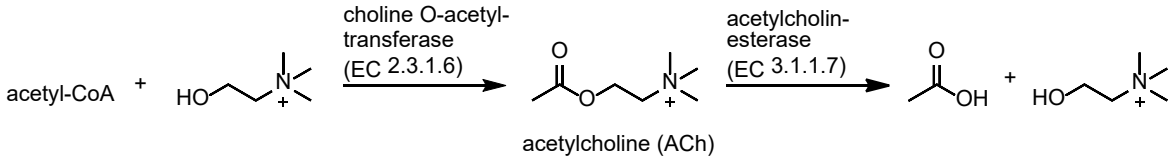


Figure 1.3. Schematic illustration of the enzymatic formation and degradation of acetylcholine (ACh). Ach acts as agonist at muscarinic receptors (GPCRs) and nicotinic receptors (ligand-gated cation channels).

Initially, several naturally occurring ligands addressing the muscarinic acetylcholine receptors were described, including the agonists muscarine (a toxin from the mushroom *Aminita muscaria*; the family of MRs was named after this toxin) and pilocarpine (therapeutic agent for glaucoma from the *Pilocarpus* genus belonging to the family *Rutacea*) as well as antagonists such as atropine (from *Atropa belladonna*).

Muscarinic receptors are widely distributed in peripheral organs and in the central nervous system (CNS). In the CNS, MRs exert neuromodulatory functions. In the periphery, they are a key component of the parasympathetic nervous system, regulating the function of various organs and glands (*cf.* Table 1.1). Moreover, MRs were reported to be involved in the regulation of cell proliferation and to be expressed on human immune cells, suggesting a modulatory role of MRs in inflammatory and immune response.[35, 40]

Consequently, MRs have emerged as attractive and important drug targets for the treatments of numerous diseases such as chronic obstructive pulmonary disease (COPD), asthma, abdominal spasms, overactive bladder, glaucoma and neurological disorders, such as AD, Parkinson's disease (PD), drug addiction, depression or schizophrenia (SZ).[35, 41-47] It should be mentioned, that the neuromodulatory effects mediated by muscarinic receptors have not been unequivocally assigned to the individual subtypes due to a lack of highly subtype selective MR agonists and antagonists, which are needed as pharmacological tools, as well as co-expression of several subtypes in the same brain regions. For this reason, the list of MR modulated physiological functions, presented in Table 1.1, is incomplete.

Table 1.1. Expression and physiological functions of muscarinic acetylcholine receptors.[a]

Muscarinic receptor	Tissue expression (human)[b]	Physiological and therapeutic relevance (the latter indicated by '→')
M_1	○ CNS: e.g. cerebral cortex, striatum, basal ganglia, hypothalamus, limbic system (e.g. amygdala and hippocampus), putamen ○ periphery: e.g. eye (iris), lung, prostate, urinary bladder, vasculature	○ modulation of attention, cognition and learning processes → AD, dementia, SZ ○ mediation of bronchoconstriction ○ mediation of gastric acid secretion → gastric ulcers (M_1R antagonists)
M_2	○ CNS: widely distributed, e.g. brainstem, cerebral cortex, hypothalamus, hippocampus and thalamus ○ periphery: e.g. heart, lung, GI tract, eye (ciliary body), prostate, submandibular gland, ureter, urinary bladder	○ reduction of heart rate and cardiac contractility ○ modulation of smooth muscle contraction → glaucoma ○ autoreceptor function in the central and peripheral nervous system (modulation of ACh release) →AD, SZ (M_2R antagonists)
M_3	○ CNS: e.g. hippocampus, hypothalamus ○ periphery: e.g. blood vessels, eye (ciliary body, iris), GI tract, lung, pancreatic gland, prostate, retina, salivary gland, testis, ureter, urinary bladder	○ stimulation of saliva, gastric acid and insulin secretion → dry mouth syndrome ○ mediation of smooth muscle contraction (trachea, bronchi, ileum, iris sphincter, urinary bladder, vasculature) → COPD, overactive bladder, irritable bowel syndrome
M_4	○ CNS: e.g. striatum, amygdala, neocortex, hippocampus, hypothalamus, putamen, spinal cord ○ periphery: e.g. autonomic ganglia, eye (ciliary body, iris), urinary bladder	○ modulation of ACh release ○ modulation of dopamine release → SZ, PD
M_5	○ CNS: e.g. substantia nigra pars compacta hippocampus, hypothalamus, ventral tegmental area ○ periphery: e.g. eye (ciliary body), ureter, urinary bladder, vasculature	○ modulation of striatal dopamine release → drug addiction ○ mediation of vasorelaxation

[a]Information were taken from the following sources: Cortes *et al.*,[48] Levey *et al.*,[49] Levey *et al.*,[50] Langmead *et al.*,[51] Zhang *et al.*,[52] Nietgen *et al.*,[53] Tyagi *et al.*,[54] Wess,[55] Wess *et al.*,[47] Andersson,[34] Bolbecker *et al.*,[41] Bubser *et al.*,[56] Buels *et al.*,[57] Eglen,[35] Ehlert *et al.*,[58] Harvey,[59] Mitchelson,[60] McDonald *et al.*,[61] Sellers *et al.*,[62] Nishtala *et al.*,[63] Clader *et al.*,[64] Lebois *et al.*,[65] Raffa..

[b]Expression analysis was based on the identification of mRNA, on autoradiography using radiolabeled receptor ligands or on immunocytochemistry using antibodies.

[c]Given are well understood physiological functions. Note: MRs are involved in the regulation of further physiological processes, such as thermoregulation, nociception and circadian rhythm, but an unambiguous subtype-specific elucidation of these mechanisms has not been achieved to date.

1.2.2. MR crystal structures and the dualsteric/bitopic ligand approach

Among the five muscarinic acetylcholine receptor subtypes, the orthosteric (acetylcholine) binding pocket is highly conserved. Thus, the development of highly subtype-selective MR ligands has been very challenging and potent and selective therapeutics without side effects (attributed to actions at undesired MR subtypes) are still an unfulfilled need. Over the past

few years, X-ray or cryo-EM crystal structures for all five subtypes of human muscarinic acetylcholine receptors were reported (Table 1.2).

Table 1.2. Crystal or cryo-EM structures of muscarinic acetylcholine receptors.

MR subtype	Activation state	Bound ligand	Ref.
M_1R	inactive	tiotropium (antagonist)	a
	active	iperoxo (agonist) (cryo-EM structure)	b
M_2R	inactive	QNB (antagonist)	c
	inactive	NMS (antagonist)	d
	inactive	AF-DX 384 (antagonist)	d
	active	iperoxo (agonist)	e
	active	iperoxo (agonist) and allosteric modulator LY2119620	e
	active	iperoxo (agonist) (cryo-EM structure)	b
M_3R	inactive	BS46 (antagonist)	f
	inactive	tiotropium (antagonist)	g,h
	inactive	NMS (antagonist)	h
M_4R	inactive	tiotropium (antagonist)	a
M_5R	inactive	tiotropium (antagonist, inverse agonist)	i

References are as follows: (a) Thal *et al.*;[67] (b) Maeda *et al.*;[68] (c) Haga *et al.*;[69] (d) Suno *et al.*;[70] (e) Kruse *et al.*;[71] (f) Liu *et al.*;[72] (g) Kruse *et al.*;[73] (h) Thorsen et al.;[74] (i) Vuckovic et al.[75]

These crystal structures confirmed the high structural similarity of MRs within the 7-TM bundle harbouring the orthosteric binding site, a phenomenon that had earlier been concluded from the high sequence homology among MRs within the transmembrane domains and from poorly pronounced selectivity profiles of orthosteric MR ligands. On top of the orthosteric binding site, i.e. near the receptor surface, MRs exhibit, as many other GPCRs, another pocket, referred to as the receptor vestibule. In the case of MRs, this well-shaped vestibule is often called the common allosteric site, which is less conserved compared to the orthosteric binding pocket.[73, 76] Therefore, the development of allosteric MR ligands or modulators has been seen as a promising approach to overcome the lack of highly selective MR ligands. Indeed, this gave rise to use MRs as model receptors to study allosterism at GPCRs.[46, 77-81] Binding of allosteric modulators induces a change in receptor conformation, resulting in a modulation of the binding affinity (and eventually also potency and efficacy) of orthosteric ligands.[82]

In 1976 gallamine was identified as the first negative allosteric modulator of muscarinic acetylcholine receptors, inhibiting the action of acetylcholine and carbachol.[83] Since then, a wide variety of allosteric MR modulators with moderate to high MR subtype selectivity has been discovered, most of them interacting with the receptor within the common allosteric site.[76] The major challenge regarding the development of allosteric MR ligands seems to be high receptor affinity as most described allosteric MR ligands exhibit rather low receptor affinity, with dissociation constants > 0.1 μM.[12] This issue can be addressed by designing

dualsteric (bitopic) ligands, which occupy the orthosteric site and (in part) also the less conserved common allosteric site, a strategy called the dualsteric ligand approach.[46, 78, 84-88] Interactions within the orthosteric pocket should contribute to high receptor affinity and interactions within the allosteric site are anticipated to mediate subtype selectivity.

1.3. Subtype preferring or selective MR ligands

1.3.1. General aspects

A multitude of therapeutic drugs addressing MRs have been identified over the past decades, but many of these ligands show no or only low subtype selectivity, causing adverse effects.[56, 57, 62] Interestingly, the lack of subtype selective MR ligands seems to give justification for calling ligands “selective”, which would have been declared as “subtype preferring” or “non-selective” outside the muscarinic receptor field. For illustration, two examples are given in the following:

(*1*) In many scientific reports pirenzepine is called an M_1R selective antagonist and has been used as such in pharmacological studies. However, with pK_i values of 8.0 (M_1), 6.3 (M_2), 6.8 (M_3), 7.0 (M_4), 6.9 (M_5), corresponding to only 10-fold higher M_1R affinity compared to the M_4R and M_5R, one should question if M_1Rs can be selectively blocked by this compound (*cf*. Figure 1.4).[62]

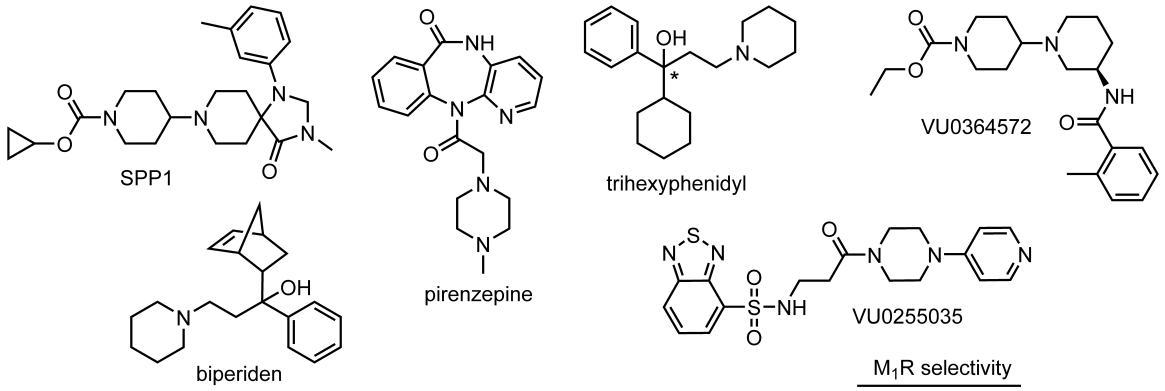
(*2*) The so-called M_2R selective antagonist AF-DX 384 exhibits the following selectivity profile (pK_i values): 7.51 (M_1), 8.22 (M_2), 7.18 (M_3), 8.0 (M_4), 6.27 (M_5).[89] It is obvious that AF-DX 384 does not discriminate between the M_2 and M_4 muscarinic receptor subtypes. Furthermore, the M_2R affinity is only 5-fold and 11-fold higher compared to the M_1R and M_3R affinity, respectively.

Several MR ligands, mainly representing peripherally acting MR antagonists (antimuscarinics), are in clinical use, e.g. ipratropium (Berodual®) and tiotropium (Spiriva®) to treat asthma and COPD, or butylscopolamine (Buscopan®) to treat abdominal spasms. The lack of highly selective MR orthosteric ligands demands alternative approaches to enhance subtype selectivity in order to reduce side effects. Therefore, there is a need for highly subtype selective MR agonists and antagonists in order to improve established clinical applications as well as to pave the way to new therapeutic approaches.

In the following, for each MR subtype, the most selective reported MR ligands are briefly discussed.

1.3.2. M_1 receptor preferring ligands

In 2019, spiropiperidine 1 (SPP1) was developed and presented as a potent partial M_1R orthosteric agonist with selectivity according to results from functional assays.[12, 90] The selectivity of the brain penetrant M_1R ligand SPP1 was assessed *in vitro* in functional assays in relevant tissues (rat atrium (M_2R), rat ileum (M_3R)), where SPP1 was devoid of agonistic effects, but showed antagonistic effects by blocking the antagonist carbachol in these tissues.[90] However, in terms of MR binding, SPP1 only slightly prefers the M_1R over the M_2, M_4 and M_5 receptor (*cf*. Figure 1.4):[90]



			pK_i					M_1R selectivity ratios K_i ($M_{2,3,4,5}R$)/K_i (M_1R)			
Compd.	Type	Ref.	M_1	M_2	M_3	M_4	M_5	M_2	M_3	M_4	M_5
SPP1	A	a*	**7.67**	6.89	5.20	6.94	6.71	6.0	370	5.4	9.2
biperiden	Ant	b*	**9.32**	8.20	8.41	8.62	8.20	13	8.1	5.0	13
pirenzepine	Ant	c	**8.0**	6.3	6.8	7.0	6.9	50	16	10	16
(*R*)-trihexyphenidyl	Ant	d	**9.43**	8.15	8.61	9.08	8.30	19	6.6	2.2	13
VU255035	Ant	e*	**7.83**	6.18	6.06	5.93	5.63	44	59	79	159
VU0364572	AM	f	-	-	-	-	-	-	-	-	-

Figure 1.4. Chemical structures of M_1R preferring ligands (A = agonist; Ant = antagonist; AM = allosteric modulator) reported in literature. References are as follows: (a) Broad *et al*.;[90] (b) Bolden *et al*.;[91] (c) Sellers *et al*.;[62] (d) Dörje et al.,[89] note: only binding data (pK_i) of the (*R*)-enantiomere are shown; (e) Sheffler *et al*.;[92] (f) Lebois *et al*.;[93] *reported K_i or K_d values were converted to pK_i or pK_d values; note: in the case of AMs, the determination of binding data by competition binding with labeled orthosteric ligands is not feasible.

Prior to this, the development of selective orthosteric M_1R ligands had been unsuccessful. Therefore, the design of M_1R selective ligands has focused on targeting a less conserved allosteric binding site or aiming at a bitopic binding mechanism at the M_1R. This yielded ligands with selectivity according to results of functional assays, however, binding data were not provided in these studies.[93-95] The most promising compound of this series was VU0364572 (*cf*. Figure 1.4), a bitopic M_1R agonist, with almost no functional activity at the other MR subtypes (EC_{50} (M_1R) = 110 nM, EC_{50} (M_2-M_5R) > 30 µM), however, radioligand competition binding data were again not provided.[93]

Several non-selective MR agonists, including cevimeline, milameline, sabcomeline, talsaclidine and xanomeline were developed for the treatment of AD, in which the

therapeutic effects were considered to be mediated by M_1R activation in the CNS. However, due to the promiscuity of these orthosteric MR agonists with respect to MR binding, the roles of the individual MR subtypes in observed *in vivo* effects remain unclear.[56] All of these compounds reached various stages of clinical trials for the treatment of AD, however, due to their poor subtype selectivity and associated cholinergic side effects, e.g. nausea and diarrhoea, the clinical trials exhibited a high drop-out rate, limiting the administrable doses of the drug candidates.[51, 96] Cevimeline (Evoxac®) (K_i $M_1R/M_2R/M_3R/M_4R$ = 1:0.18:0.53:0.21),[97] which was currently approved by the FDA for the treatment of xerostomia (dry mouth) associated with Sjögren's syndrome, still exhibits several cholinergic side effects due to the lacking subtype selectivity.[98]

Likewise, there is also a lack of highly selective M_1R antagonists. In 2009, VU0255035 was introduced as a selective orthosteric M_1 receptor antagonist, reducing pilocarpine-induced seizures in mice.[92] This ligand exhibited a rather good M_1R selectivity (Figure 1.4),[92] and was therefore further investigated in several studies with respect to its potential clinical use.[99-101]

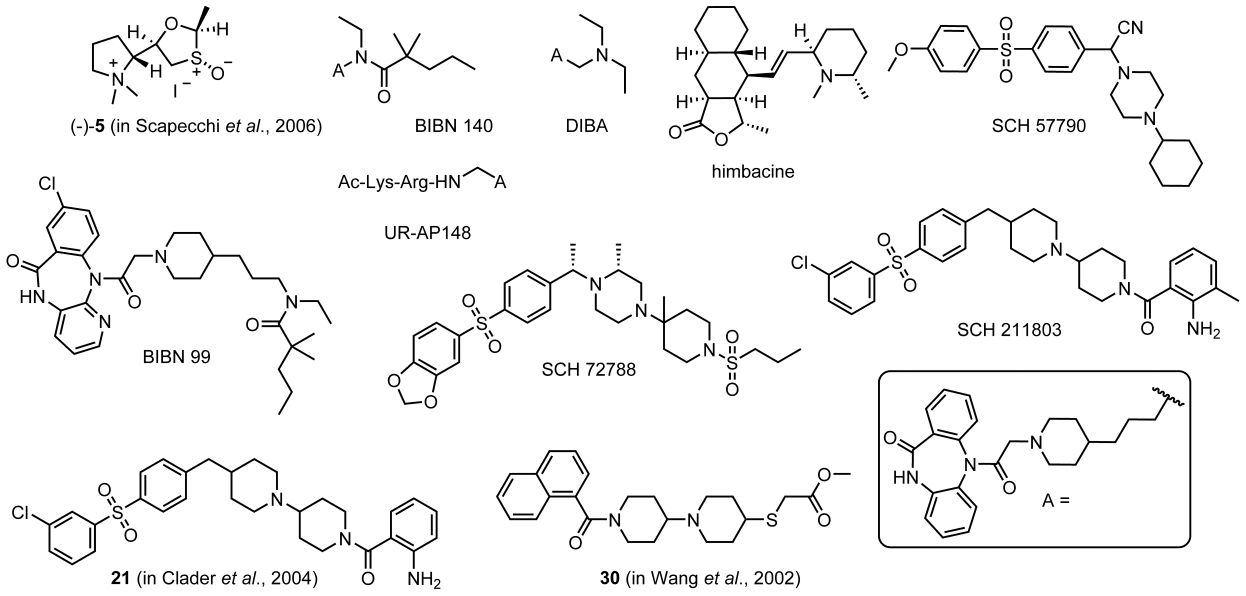
As mentioned above, the so called M_1R selective antagonist pirenzepine (Gastrozepin®), which is used in the treatment of peptic, gastric and duodenal ulcers, shows only a 10 to 16-fold higher M_1R affinity compared to the M_3R, M_4R and M_5R (Figure 1.4). Likewise, the clinically approved MR ligands biperiden (Akineton®) and trihexyphenidyl (Artane®) act as antagonists at M_1R and M_4R, showing a slight selectivity for M_1R, and are used to relieve smooth muscle tone, sweating, salvation and to reduce rigor and tremor in patients with Parkinsonism.[102] Biperiden only shows up to 13-fold higher affinity to the M_1R, which causes several cholinergic side effects such as nausea, constipation, gastric irritation, urinary retention or dry mouth due to antagonism at other MR subtypes.

To date, no highly selective M_1R agonists and antagonist are available as licensed drugs.

1.3.3. M_2 receptor preferring ligands

Whereas the search for selective M_2R agonists has been neglected (compound (−)-**5** (in Scapecchi et al.) is the only reported agonist showing a preference for M_2R over the other four subtypes),[103] several attempts have been made to design highly selective M_2R antagonists (Figure 1.5). Early discoveries were based on natural products, dibenzodiazepinones, piperazines, and piperidines. The dibenzodiazepinone DIBA and the pyridobenzodiazepinone BIBN 99, both acting as M_2R antagonists, showed high M_2R affinity and moderate M_2R selectivity over the other muscarinic receptors (Figure 1.5), representing privileged scaffolds to develop selective M_2R antagonists.[104, 105] Thereupon,

several compounds derived from DIBA or BIBN 99 were developed and characterized, but only few ligands showed a slightly improved M_2R selectivity, for instance BIBN 140.[106-111] Himbacine, an alkaloid isolated from the bark of Australian magnolias, exhibits high M_2R affinity (pK_i = 8.00) and modest M_2R selectivity (*cf*. Figure 1.5), rendering himbacine a promising starting point in AD research (mode of action: selective antagonism at M_2



			pK_i / pK_d					M_2R selectivity ratios K_i ($M_{1,3,4,5}R$)/K_i (M_2R)			
Compd.	Type	Ref.	M_1	M_2	M_3	M_4	M_5	M_1	M_3	M_4	M_5
(-)-**5** (in Scapecchi *et al*., 2006)	A	a	< 4	**5.03**	< 4	< 4	< 4	> 11	> 11	> 11	> 11
21 (in Clader *et al*., 2004)	Ant	b	n.a.	**9.70**	n.a.	n.a.	n.a.	2926	2722	148	186
30 (in Wang *et al*., 2002)	Ant	c*	n.a.	**9.15**	n.a.	n.a.	n.a.	1400	1500	57	400
BIBN 99	Ant	d	5.97	**7.52**	6.11	6.76	5.84	35	26	5.8	48
BIBN 140	Ant	e	6.5	**8.1**	6.4	7.3	6.5	40	50	6.3	40
DIBA	Ant	f*	8.4	**9.5**	8.0	8.7	n.a.	13	37	7.0	-
himbacine	Ant	g	6.97	**8.00**	7.03	7.96	6.31	11	9.3	1.1	49
SCH 57790	Ant	h*	6.95	**8.04**	7.10	7.42	n.a.	13	8.8	4.2	-
SCH 72788	Ant	i*	7.38	**9.30**	7.64	8.52	7.46	84	46	6	70
SCH 211803	Ant	c*	n.a.	**8.95**	n.a.	n.a.	n.a.	730	790	69	95
UR-AP148	Ant	j	7.38	**8.97**	5.17	7.21	6.37	49	6500	60	400

Figure 1.5. Chemical structures of M_2R preferring ligands (A = agonist; Ant = antagonist) reported in literature. References are as follows: (a) Scapecchi et al.,[103] (b) Clader *et al*.,[112] note: K_i values for MR subtypes M_1, M_3, M_4 and M_5 were not provided; (c) Wang *et al*.,[113] note: K_i values for MR subtypes M_1, M_3, M_4 and M_5 were not provided; (d) Doods *et al*.,[104] (e) Doods *et al*.,[114] (f) Gitler *et al*.,[105] (g) Dörje *et al*.,[89] (h) Lachowicz *et al*.,[115] (i) Lachowicz *et al.,*[116] (j) Pegoli et al.;[117] n.a. not analyzed, *reported K_i or K_d values were converted to pK_i or pK_d values.

autoreceptors in the CNS).[89] However, the development of M_2R selective antagonists, derived from himbacine, failed.

SCH 57790 was the first compound of a new class of piperazine-based M_2R preferring compounds, presented in 2001.[115] The incorporation of a terminal piperidine moiety resulted in the discovery of SCH 72788, which exhibited an improved M_2R selectivity over M_1R, M_3R and M_5R (*cf*. Figure 1.5).[116] Another structural modulation of SCH 57790 led to the

piperidinylpiperidine analogue SCH 211803, showing an enhanced M_2R selectivity, which had entered phase 1 clinical trials.[113, 118] Based on SCH 211803, several compounds were developed and characterized, i.e. compound **30** (in Wang *et al.*)[113] and **21** (in Clader *et al.*)[112], showing the best M_2R selectivity profiles in this series.

Using the dualsteric ligand approach, a series of dibenzodiazepinone derivatives, containing a short peptide, was developed, where the peptide moiety, supposed to interact with the receptor *via* allosteric sites, was shown to modulate MR subtype selectivity.[117] UR-AP148 (**3**) exhibited the highest M_2R selectivity in this series (Figure 1.5) and is further discussed in chapter 2.

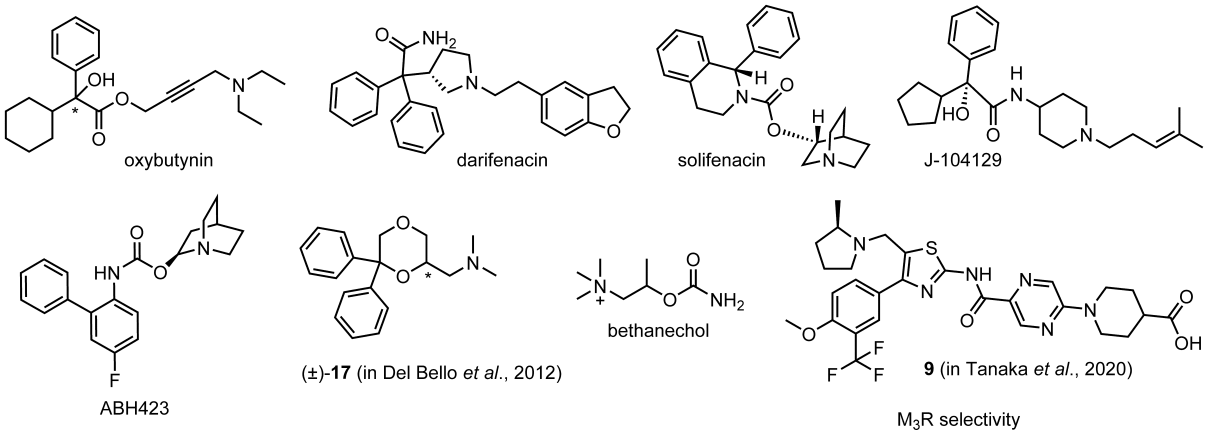
As antagonism at presynaptic M_2Rs represents an alternative approach to increase cholinergic transmission by increased ACh levels in patients with, e.g. AD or SZ, but selective M_2R ligands are not yet available as licensed drugs, there is still a need for highly selective M_2R antagonists.

1.3.4. M_3 receptor preferring ligands

Although potent anticholinergics for the treatment of, e.g. COPD or overactive bladder (OAB), are being therapeutically used, most have little subtype selectivity. Oxybutynin (Dridase®), darifenacin (Emselex®) and solifenacin (Vesikur®), used for the treatment of OAB, are the only licensed drugs, exhibiting a certain M_3R selectivity.

The M_3R antagonist J-104129 shows a high degree of selectivity for the M_3R over M_2R (Figure 1.6).[119] However, the bronchodilating activity of J-104129, when applied orally in COPD patients, was inferior to the dilating effect achieved by inhalation of the rather unselective compound ipratropium bromide, and additional side effects like dry mouth were more pronounced in the J-104129 treatment group of a 6-week phase II study comprising 412 COPD patients.[120] This study demonstrated, that high M_3R over M_2R selectivity of an oral antimuscarinic agent is not enough to improve the therapeutic effect in patients with COPD compared with inhaled non-selective antimuscarinics.[121] The experimental ligand ABH423 (Figure 1.6) derived from the non-selective antagonist 3-quinuclidinylbenzilate (QNB) showed 91-fold selectivity over the M_2R and was further modified in various positions, yielding ligands exhibiting 46- to 68-fold M_3R over M_2R selectivity (data not shown).[122]

The tertiary amine (±)-**17** (in Del Bello *et al.*), derived from 1,4-dioxane, preferred M_3R by approx. 2-fold over $M_{1,4,5}R$ and by approx. 8-fold over M_2R and proved to be effective in reducing the contraction of rat urinary bladder (Figure 1.6)[123] Moreover, highly selective M_3R agonists have not been described as well. Bethanechol (Myocholine-Glenwood®), which stimulates contraction of the bladder and expulsion of urine, is the only M_3R agonist showing a slight selectivity for M_3R (Figure 1.6).[38]



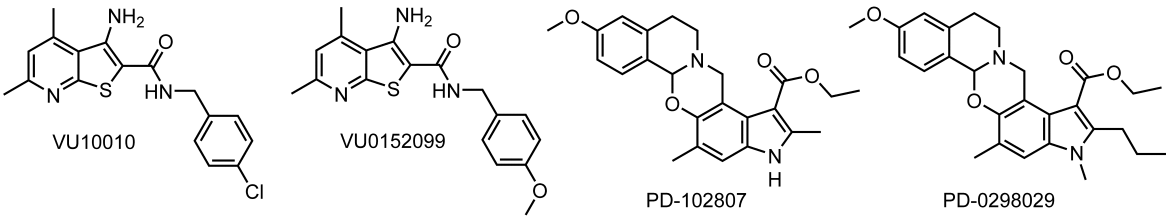
			pK_i					M_3R selectivity ratios K_i ($M_{1,2,4,5}$R)/K_i (M_3R)			
Compd.	Type	Ref.	M_1	M_2	M_3	M_4	M_5	M_1	M_2	M_4	M_5
9 (in Tanaka *et al*., 2020)	AM	a	-	-	-	-	-	-	-	-	-
(±)-**17** (in Del Bello *et al*., 2012)	Ant	b	8.32	7.78	**8.66**	8.32	8.27	2.1	7.6	2.2	2.5
ABH423	Ant	c	9.28	7.50	**9.47**	n.a.	n.a.	1.6	91	-	-
bethanechol	A	d	4.01	4..4	**4.15**	4.03	n.a.	1.4	1.3	1.3	-
darifenacin	Ant	e	8.2	7.4	**9.1**	7.3	8.0	7.9	50	63	13
J-104129	Ant	f*	7.60	6.12	**8.19**	n.a	n.a.	3.8	120	-	-
oxybutynin	Ant	e	8.7	7.8	**8.9**	8.0	7.4	1.6	13	7.9	32
solifenacin	Ant	g	7.6	6.9	**8.0**	n.a.	n.a.	2.5	13	-	-

Figure 1.6. Chemical structures of M_3R preferring ligands (A = agonist; Ant = antagonist; AM = allosteric modulator) reported in literature. References are as follows: (a) Tanaka *et al*.,[124] (b) Scapecchi et al.,[103] (c) Fischer *et al*.,[122] note: K_i values for MR subtypes M_4 and M_5 were not provided; (d) Jakubik *et al*.,[38] note: K_i value for M_5R was not provided; (e) Abrams *et al*.,[125] (f) Mitsuya *et al*.,[119] (g) Ikeda *et al*.;[126] n.a. not analyzed; *reported K_i values were converted to pK_i values. note: in the case of AMs, the determination of binding data by competition binding with labeled orthosteric ligands is not feasible.

In 2020, the positive allosteric modulator (PAM) **9** (in Tanaka *et al*.) was discovered (for structure see Figure 1.6), showing moderate to high subtype selectivity over the other MR subtypes, which was assessed by the Carbachol (CCh) dependent Ca^{2+} increase in functional assays.[124] When used at a concentration of 10 µM, compound **9** (in Tanaka *et al*.) caused a 269-fold decrease in the EC_{50} of CCh (M_3R activation), whereas the decrease in the EC_{50} of CCh was lower in the case of other MR subtypes (M_1R: 2.3-fold, M_2R: 1.3-fold, M_4R: 1.0-fold, M_5R: 94-fold).[124]

1.3.5. M_4 receptor preferring ligands

To date, no orthosteric selective M_4R agonists, which might be useful for treating positive symptoms (e.g. hallucinations, delusions) of SZ associated with hyperdopaminergia,[127-129] have been discovered. However, activation of the muscarinic M_4 receptor can also be mediated by allosteric ligands. The positive allosteric modulator (PAM) VU10010 represented a breakthrough when described in 2008 (Figure 1.7).[130] When used at a concentration of 10 µM, VU10010 induced a 47-fold potentiation of M_4R ACh potency, whereas the response to ACh in cell lines expressing each of the other MR subtypes was unaffected.[130]



			pK_i					M_4R selectivity ratios K_i ($M_{1,2,3,5}$R)/K_i (M_4R)			
Compd.	Type	Ref.	M_1	M_2	M_3	M_4	M_5	M_1	M_2	M_3	M_5
PD-102807	Ant	a	5.88	6.27	6.59	**8.15**	5.46	190	76	36	480
PD-0298029	Ant	a	4.88	6.47	6.82	**9.00**	4.95	13000	340	150	11000
VU10010	AM	b	-	-	-	**-**	-	-	-	-	-
VU0152099	AM	c	-	-	-	**-**	-	-	-	-	-

Figure 1.7. Chemical structures of M_4R preferring ligands (Ant = antagonist; AM = allosteric modulator) reported in literature. References are as follows: (a) Böhme *et al*.[131] (b) Shirey *et al*.,[130] (c) Brady *et al*.;[132] note: in the case of AMs, the determination of binding data by competition binding with labeled orthosteric ligands is not feasible.

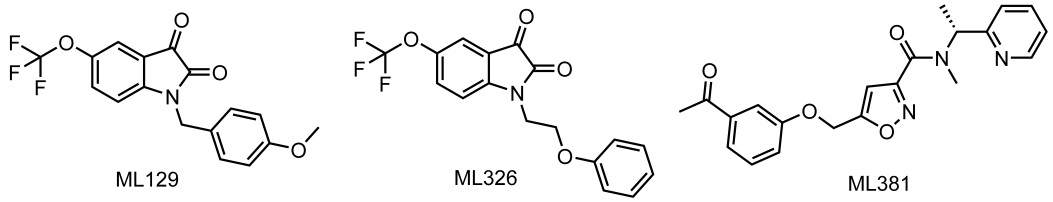
Subsequent optimization approaches led to the discovery of VU0152100, which induced a dose-dependent shift in ACh potency (maximal increase over control: 70-fold).[132]

On the other hand, also the development of selective M_4R antagonists, which were suggested to restore the dopamine acetylcholine balance in patients with PD, has been challenging.[133] To date, only PD-102807, PD-0298029 and a few other benzoxazine analogues were described (Figure 1.7).[131] The former is still used in scientific research for studying the effects of the different MR subtypes in the brain and in the periphery.[134-136] PD-0298029 exhibited poor bioavailability and rapid metabolism in animal studies, which limits its use to *in vitro* studies.[137]

In summary, selective M_4R ligands, representing drug candidates, e.g. for the treatment of AD, SZ or PD, are not available to date.

1.3.6. M_5 receptor preferring ligands

To date, no selective M_5R agonists have been discovered, however, a series of M_5R positive allosteric modulators was described (Figure 1.8). The first PAM for the M_5R, ML129, was reported in 2009, provoking higher potentiation of ACh induced intracellular Ca^{2+}-mobilization at the M_5R compared to the M_1R-M_4R (EC_{50} values of ACh in the presence of ML129: M_1R-M_4R: >30 µM, M_5R: 1.16 µM).[138] Only a few years later, ML326 was discovered as the first sub-micromolar, selective M_5R PAM (EC_{50} values of ACh in the presence of ML326: M_1R-M_4R: >30 µM, M_5R: 410 nM) (Figure 1.8).[139]



			pK_i				
Compd.	Type	Ref.	M_1	M_2	M_3	M_4	M_5
ML129	AM	a	-	-	-	-	-
ML326	AM	b	-	-	-	-	-
ML381	Ant	c	-	-	-	-	6.47

Figure 1.8 Chemical structures of the positive allosteric M_5R modulators ML129 and ML326 and the M_5R antagonist ML381 (Ant = antagonist; AM = allosteric modulator). References are as follows: (a) Bridges *et al*.,[138] (b) Gentry *et al*.,[139] (c) Gentry *et al*.;[140] note: in the case of AMs, the determination of binding data by competition binding with labeled orthosteric ligands is not feasible.

Shortly thereafter, in 2014, the orthosteric M_5R antagonist ML381 was reported (Figure 1.8). According to the results from functional studies, i.e. the inhibition of ACh induced Ca^{2+} response, this compound displayed M_5R selective antagonism (IC_{50} (M_1R) = >10 µM; IC_{50} (M_2R-M_4R) = >30 µM; IC_{50} (M_5R) = 0.45 µM). However, MR binding data of ML381 were only reported for the M_5R (Figure 1.8).[140]

1.4. Scope and objectives

Muscarinic acetylcholine receptors (MRs) are widely distributed in the central and peripheral nervous system, being (potential) targets for the treatment of various diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), schizophrenia (SZ), overactive bladder (OAB) or chronic obstructive pulmonary disease (COPD). Due to the high conservation of the orthosteric binding pocket within the family of MRs, the development of highly selective MR ligands has been extremely challenging, and to date, no MR ligands, which are selective for one of the five subtypes, are available as licensed drugs. Highly subtype-selective MR ligands are needed as new therapeutics with lower side effects, but also as molecular tools required, e.g. for pharmacological studies. As the vestibule (common allosteric binding site) of MRs is less conserved than the orthosteric binding pocket, the dualsteric ligand approach, i.e. the design of ligands interacting with both, the orthosteric and the allosteric binding site, has been considered useful for the development of highly subtype selective MR ligands,[84-88] This approach benefits from recently reported MR crystal structures, which are available for all MR subtypes.[67-75]

Lately, conjugation of small peptides to the M_2R preferring dibenzodiazepinone-type MR antagonist DIBA (referred to as DIBA-peptide conjugates) was shown to considerably influence M_2R selectivity.[117] The introduction of basic amino acids into the peptide moiety,

which is supposed to interact with the receptor within the common allosteric site or at the surface of the receptor, was advantageous with respect to improved M_2R selectivity compared to non-peptidic tricyclic MR antagonists.[117]

For the synthesis of the recently reported series of DIBA-peptide conjugates, only proteinogenic amino acids and only two different linkers were used.[117] In this work, the novel approach had to be further explored by using a broad variety of linkers, connecting the DIBA pharmacophore and the peptide moiety, and by incorporating unnatural amino acids into the peptide moiety. The design of the presented DIBA-peptide conjugates, presented in this work, had to be supported by induced-fit docking studies. MR affinities of the new DIBA-peptide conjugates had to be determined for the subtypes M_1, M_2 and M_4 in radioligand competition binding assays at intact CHO cells stably expressing the respective receptors. For selected compounds, showing high M_2R selectivity over the M_1 and M_4 receptor, binding to the M_3 and M_5 receptor had to be also investigated, resulting in complete selectivity profiles. In order to study the mode of action at the M_2R (anticipated to be antagonistic activity), a few DIBA-peptide conjugates, exhibiting high M_2R selectivity, had to be investigated in a functional M_2R assay. Moreover, selected DIBA-peptide conjugates had to be investigated in terms of stability in human plasma/PBS.

Finally, a series of fluorescently labeled dibenzodiazepinone-type MR ligands had to be prepared using various fluorescent dyes (5-TAMRA, $\lambda_{ex}/\lambda_{em}$ ≈ 547/576 nm; BODIPY 630/650, $\lambda_{ex}/\lambda_{em}$ ≈ 625/640 nm; pyridinium dye Py-1, $\lambda_{ex}/\lambda_{em}$ ≈ 611/665 nm and pyridinium dye Py-5, $\lambda_{ex}/\lambda_{em}$ ≈ 465/732 nm). M_1-M_5 receptor affinities of the fluorescent probes had to be determined by radioligand competition binding and their applicability for fluorescence-based methods had to be explored by performing flow cytometric saturation binding experiments at CHO-hM_2R cells.

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CHAPTER 2

Dibenzodiazepinone-type muscarinic receptor antagonists conjugated to basic peptides: impact of the linker moiety and unnatural amino acids on M_2R selectivity

Prior to the submission of this thesis, this chapter was published in cooperation with partners:

Corinna G. Weinhart, David Wifling, Maximilian F. Schmidt, Eduard Neu, Carina Hörng, Timothy Clark, Peter Gmeiner and Max Keller. Dibenzodiazepinone-type muscarinic receptor antagonists conjugated to basic peptides: impact of the linker moiety and unnatural amino acids on M_2R selectivity. *Eur. J. Med. Chem*., **2020**, submitted. Reproduced with permission from Eur. J. Med. Chem.

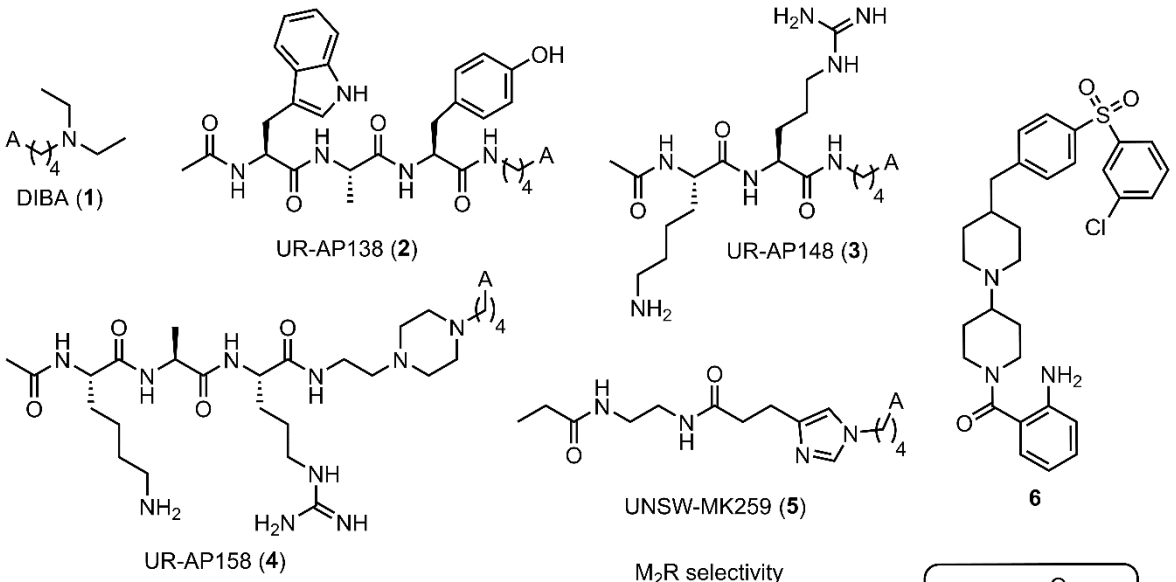
Author contributions:

C.G.W. synthesized the ligands. C.G.W performed radiochemical binding and functional experiments and analysed the data. D.W., M.F.S and E.N. performed induced fit docking studies. C.H. generated utilized plasmids and HEK293T cells expressing NlucN-mini-G_{si} and M_2R-NlucC constructs. M.K. initiated the project and supervised the research. C.G.W. and M.K. wrote the manuscript. All authors have given approval to the final version of the manuscript.

2.1. Introduction

In humans, muscarinic acetylcholine receptors (MRs), belonging to class A of G-protein-coupled receptors (GPCRs), comprise five highly homologues subtypes (M_1R-M_5R).[1] Whereas the M_1, M_3 and M_5R couple to G_q-type G-proteins, the M_2 and M_4 subtypes preferentially activate $G_{i/o}$-type G-proteins.[1] MRs regulate numerous physiological functions in both the central and peripheral nervous system, and represent important targets for the treatment of pathophysiological conditions such as chronic obstructive pulmonary disease, asthma or abdominal spasms.[2-5] Moreover, Alzheimer's disease, Parkinson's disease, schizophrenia, drug addiction and depression were reported to be associated with dysregulated MR expression levels or cholinergic dysfunction,[2, 6-9] but medical drugs for the treatment of these diseases, acting at central MRs, are lacking. This can be attributed, in part, to the high conservation of the orthosteric binding pocket among MRs, being the reason for the lack of MR ligands with pronounced subtype selectivity. As the vestibule of MRs, referred to as the common allosteric binding site, is less conserved compared to the orthosteric pocket,[10] the design of dualsteric MR ligands, addressing the orthosteric and the allosteric binding site, has emerged as a promising strategy to develop subtype selective MR agonists and antagonists.[2, 11-28]

Recently, it was demonstrated that the attachment of bulky moieties (e.g. pharmacophores of various muscarinic receptor ligands or fluorophores) to M_2R-preferring dibenzodiazepinone-type MR ligands is well tolerated in terms of MR binding.[29-33] This knowledge gave rise to the synthesis of a series of DIBA (**1**) derived MR antagonists containing short peptides attached to the dibenzodiazepinone pharmacophore of (Figure 2.1).[34] A few examples of such DIBA-peptide conjugates are shown in Figure 2.1 (compounds **2-4**). Whereas **3** and **4**, containing two basic amino acids, exhibited considerably higher M_2R selectivity compared to non-peptidic dibenzodiazepinone-type ligands such as **5**, the DIBA-peptide conjugate **2**, containing the non-basic aromatic amino acids Tyr and Trp, showed no M_2R selectivity towards the M_1, M_4 and M_5 receptor (Figure 2.1). Apparently, the presence of basic amino acids is advantageous, however, it does not guarantee high M_2R selectivity.[34] Compounds such as **3** and **4** exhibit excellent M_2R selectivity towards the M_3 and M_5 receptor and moderate to high M_2R selectivity over M_1 and M_4 receptors. Higher M_2R selectivity over the M_1 and M_4 receptor was only reported for one compound class, i.e. diaryl sulfone-type MR ligands like **6** (Figure 2.1).[36]



Compd.	Ref.	pK_i M_1	M_2	M_3	M_4	M_5	M_2R selectivity ratios K_i ($M_{1,3,4,5}$R)/K_i (M_2R) M_1	M_3	M_4	M_5
1	a	8.4	9.5	8.0	8.7	-	13	37	7.0	-
2	b	7.0	7.7	5.9	7.4	6.9	4.7	56	1.9	6.2
3	b	7.4	9.0	5.2	7.2	6.4	49	6500	60	400
4	b	8.6	10	7.0	8.7	6.5	45	1800	40	4900
5	c	8.1	9.1	7.2	8.6	6.8	11	79	3.0	230
6	d	-	9.7	-	-	-	2900	2700	150	190

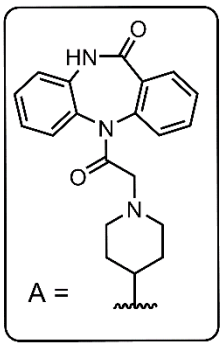


Figure 2.1. Structures and MR binding data of reported tricyclic MR antagonists (compounds **1**-**5**) and a diarylsulfone-type M_2R-selective ligand (**6**). References in the table are as follows: (a) Gitler *et. al*. (reported K_i values were converted to pK_i values)[35] (b) Pegoli *et. al.*[34] (c) Keller *et. al*. (data, reported as pIC_{50}, were reanalyzed to obtain pK_i values)[30] (d) Clader *et. al*. (the reported K_i value (M_2R) of **6** was converted to a pK_i value; note: IC_{50}, pIC_{50}; K_i, or pK_i values for the MR subtypes M_1, M_3, M_4, and M_5 were not provided; instead only the ratios K_i($M_{1,3,4,5}$R)/K_i(M_2R) were given).[36]

The recently reported DIBA-peptide conjugates contained only natural amino acids and merely two types of linkers were used to connect the DIBA pharmacophore with the peptide. In the present study, we describe a new series of DIBA-peptide conjugates all containing at least one basic amino acid. In order to investigate the impact of the linker moiety on M_2R affinity and selectivity, twelve different linkers were used for the preparation of the DIBA-peptide conjugates. Moreover, unnatural amino acids such as D-configured amino acids or arginine homologues with a shortened side chain were used for compound synthesis (Figure 2.2). We also synthesized the highly M_2R selective diaryl sulfone **6**[36] (cf. Figure 2.1) and included this compound in MR binding studies. Besides the determination of M_1R-M_5R affinities, selected compounds were investigated with respect to M_2R antagonism in a mini-

G-protein recruitment assay. Finally, induced-fit docking was performed to gain insights into possible ligand binding modes and putative ligand-receptor interactions.

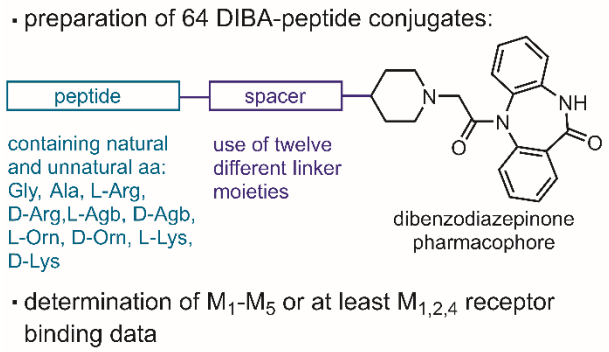


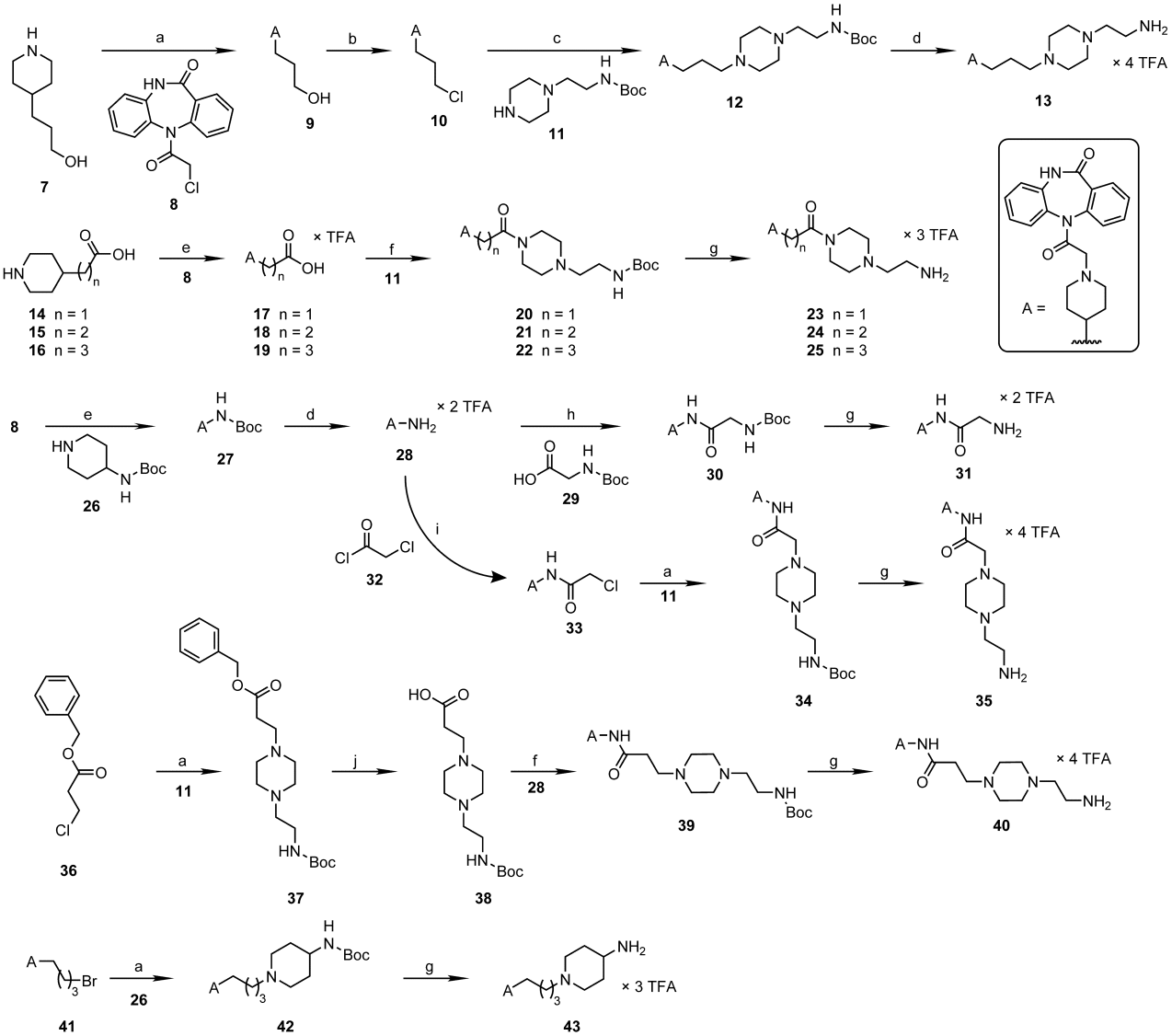
Figure 2.2. Rationale of the present study. Agb: shorter Arginine homologue with an ethylene instead of a trimethylene moiety in the side chain.

2.2. Results and Discussion

2.2.1. Chemistry

For the synthesis of the DIBA-peptide conjugates, we prepared a set of nine amine-functionalized precursor compounds containing the same dibenzodiazepinone pharmacophore, but different linker moieties (**13**, **23**-**25**, **31**, **35**, **40** and **43**) or no linker moiety (**28**) (syntheses outlined in Scheme 2.1). In addition, three previously described dibenzodiazepinone-type amine precursors (**52**, **62**, **72**; cf. Scheme 2.2) were used.[30, 33]. The synthesis of derivative **13** started with N-alkylation of piperidine **7** using the chlorinated dibenzodiazepinone derivative **8**[37] to give alcohol **9**, which was converted to chloride **10** using thionylchloride (Scheme 2.1). Nucleophilic substitution of chlorine in **10** using piperazine derivative **11**[33] gave the Boc-protected intermediate **12**, which was treated with TFA to afford precursor **13**. Amine precursors **23**-**25**, containing an acylated piperazine moiety, were prepared through N-alkylation of the commercially available piperidinyl carboxylic acids **14**-**16** by chloride **8**, giving intermediates **17**-**19**, which were used to acylate piperazine **11** using EDC × HCl/HOBt as coupling reagent. Subsequent removal of the Boc groups in the intermediates **20**-**22** gave the precursor compounds **23**-**25** (Scheme 2.1). Likewise, N-alkylation of piperidine derivative **26** using chloride **8**, followed by Boc-deprotection, afforded amine precursor **28**, which was also used to prepare precursor compounds **31** and **35**. For the synthesis of **31**, **28** was acylated with N-Boc protected glycine to give **29**, followed by Boc-deprotection of **29** yielding precursor **31**. For the synthesis of **35**, **28** was acylated using chloroacetyl chloride (**32**) to afford chloride **33**, which was used to alkylate piperazine **11**, yielding the Boc-protected intermediate **34**. Treatment

of the latter with TFA gave amine precursor **35**. The synthesis of the longer homologue of **35** (compound **40**) started with the N-alkylation of **11** using 3-chloropropionic acid benzylester (**36**) to give **37**. Debenzylation of **37** by hydrogenolysis, using 10% Pd/C and methanol as solvent, afforded carboxylic acid **38**. Amidation of **38** with amine **28** yielded the Boc-protected intermediate **39**, which was converted to amine precursor **40** by treatment with TFA. Finally, nucleophilic substitution of bromine in compound **41**[33] with amine **26** gave intermediate **42**, which was Boc-deprotected to obtain precursor compound **43** (Scheme 2.1).

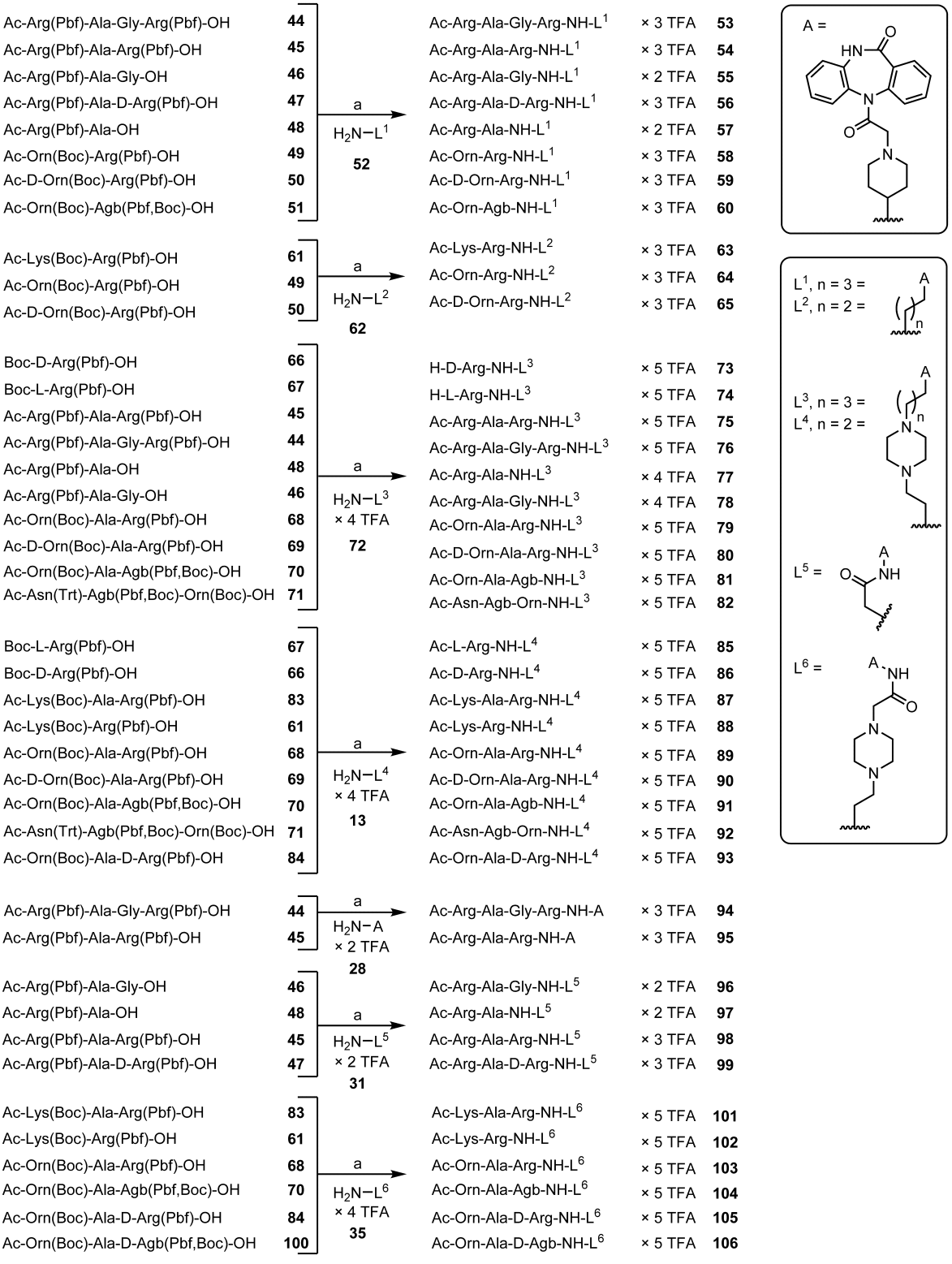


Scheme 2.1. Synthesis of the amine precursors **13**, **23**, **24**, **25**, **28**, **31**, **35**, **40** and **43**. Reagents and conditions: (a) K_2CO_3, acetonitrile, 85 °C (microwave), 90 min, 97% (**9**), 67% (**34**), 96% (**37**), 99% (**42**); (b) $SOCl_2$, pyridine, CH_2Cl_2, reflux, overnight, 99%; (c) NaI, K_2CO_3, acetone, reflux, 72 h, 82%; (d) CH_2Cl_2/TFA/H_2O 4:1:0.1 v/v/v, rt, 3 h, 67% (**13**), 87% (**28**); (e) K_2CO_3, DMF, 85 °C (microwave), 90 min, 36% (**17**), 65% (**18**), 46% (**19**), 99% (**27**); (f) EDC × HCl, HOBt, DIPEA, DMF, 55 °C, overnight, 77 % (**20**), 54% (**21**), 52% (**22**), 70% (**39**); (g) TFA/H_2O 95:5 v/v, rt, 3 h; 49% (**23**), 42% (**24**), 56% (**25**), 48%, (**31**), 53% (**35**), 61% (**40**), 89% (**43**); (h) EDC × HCl, HOBt, DIPEA, DMF, 5 °C, overnight, 60%; (i) DIPEA, DMF, rt, 2 h, 88%; (j) H_2, 10% Pd/C, MeOH, rt, 90 m in, 90%.

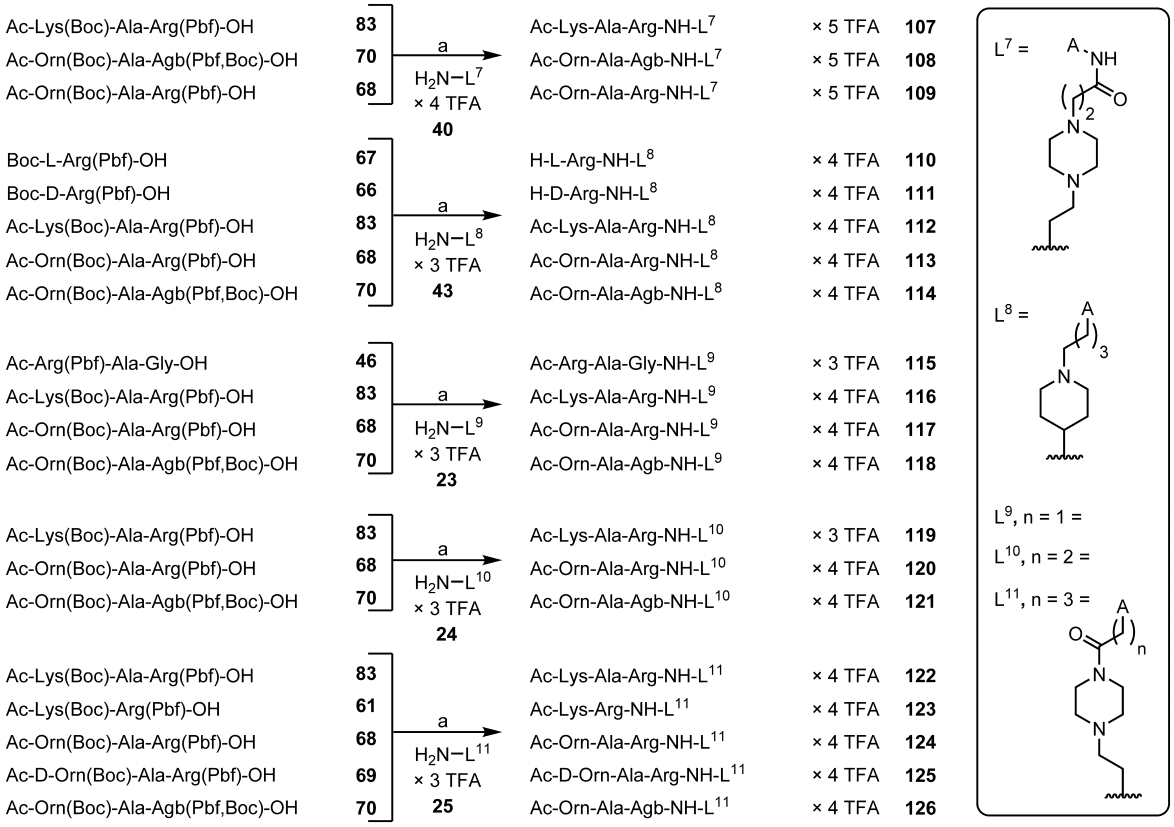
The N-terminally acetylated peptides **44**-**51**, **61**, **68**-**71**, **83**, **84** and **100** were synthesized on a chlorotrityl resin according to the Fmoc strategy using a previously described procedure.[34] For the coupling of the peptides to the amine-functionalized dibenzodiazepinone derivatives via their C-termini, EDC × HCl and HOBt were used as coupling reagents, excessive base was avoided and reactions were performed at 5 °C. As reported recently, under these conditions epimerization can be largely precluded.[34]

Peptide conjugation to amine precursors **13**, **23**-**25**, **28**, **31**, **35**, **40**, **43**, **52**, **62** or **72**, followed by cleavage of side chain protecting groups using TFA/H_2O 95:5, afforded the DIBA-peptide conjugates **53**-**60**, **63**-**65**, **73**-**82**, **87**-**99**, **101**-**109**, **112**-**126** (Schemes 2.2 and 2.3). Coupling of Boc-D-Arg(Pbf)-OH (**66**) or Boc-Arg(Pbf)-OH (**67**) to amine precursors **13**, **43** or **72**, followed by deprotection, gave compounds **73**, **74**, **85**, **86**, **110** and **111**.

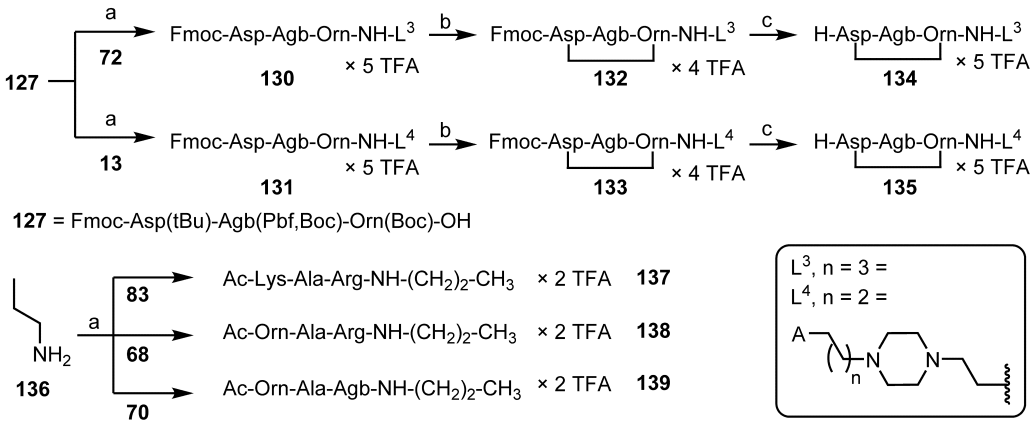
Conjugation of the N-terminally Fmoc-protected tripeptide **127** to amine precursors **13** and **72**, followed by side-chain deprotection using 95% aqueous TFA, yielded compounds **130** and **131** (Scheme 2.4). Intramolecular amide bond formation between the carboxylic acid group of the aspartic acid side chain and the amine group of the ornithine side chain in **130** and **131** was performed using PyBOP/HOBt as coupling reagents, resulting in the macrocyclic compounds **132** and **133**. Subsequent Fmoc-deprotection of **132** and **133** using diethylamine afforded the cyclic DIBA-peptide conjugates **134** and **135**. Amidation of the peptides **68**, **70** and **83** with propylamine (**136**) gave the reference compounds **137**-**139** (Scheme 2.4). We also synthesized the previously described highly M_2R selective MR ligand **6** (structure shown in Figure 2.1) according to the reported procedure.[36]



Scheme 2.2. Synthesis of the DIBA-peptide conjugates **53**-**60**, **63**-**65**, **73**-**82**, **86**-**106**. Reagents and conditions: (a) (1) EDC × HCl, HOBt, DMF, 5 °C, overnight; (2) TFA/H2O 95:5 v/v, rt, 3 h. Agb: shorter Arginine homologue with two methylene groups.



Scheme 2.3. Synthesis of the DIBA-peptide conjugates **107**-**126**. Reagents and conditions: (a) (1) EDC × HCl, HOBt, DMF, 5 °C, overnight; (2) TFA/H2O 95:5 v/v, rt, 3 h. For the structure of A see Scheme 2.1 or 2.2.

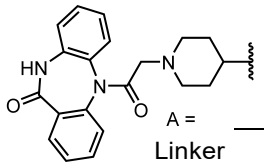


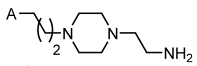
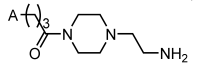
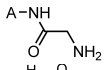
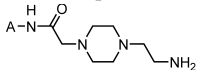
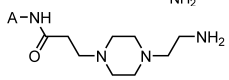
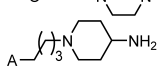
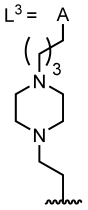
Scheme 2.4. Synthesis of the cyclic DIBA-peptide conjugates **134** and **135** and the amidated tripeptides **137**-**139**. Reagents and conditions: (a) (1) EDC × HCl, HOBt, DMF, 5 °C, overnight; (2) TFA/H_2O 95:5 v/v, rt, 3 h; (b) HOBt, DIPEA, PyBOP, DMF, rt, 5h; (c) DEA, DMF, rt, 3h. For the structure of A see Scheme 2.1 or 2.2

2.2.2. Radioligand competition binding studies with [^{3}H]NMS

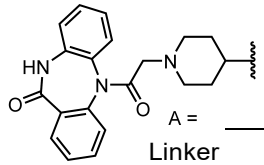
MR binding affinities were determined by radioligand competition binding at CHO cells stably transfected with the human M_1, M_2, M_3, M_4 or M_5 receptor using [^{3}H]NMS as labeled ligand. M_1, M_2 and M_4 receptor affinities were determined for all target compounds as well as amine precursors **13**, **25**, **31**, **35**, **40** and **43** because M_2R selectivity over the M_1 and M_4 receptor is most critical for the studied compound class. For the peptides **137-139**, representing reference compounds, only binding to the M_2R was investigated. M_3 and M_5 receptor affinities were additionally determined for selected compounds, showing either high M_2R selectivity compared to the M_1 and M_4 receptor (**87**, **89**, **91**, **92**, **101**, **103**-**105**, **107**-**109**, **119**, **122**) or only a low or no M_2R preference over the M_1 and M_4 receptor (**63**, **85**, **88**, **93**, **95**, **102**, **116**, **120**, **121**, **125**) (Table 2.1). Radioligand displacement curves, obtained from competition binding studies, are shown in Figure 2.3 (**89**, **95**, **103**-**105**, **109**) or Figures S1-S9 (2.5. Supplementary Information) (**13**, **25**, **31**, **35**, **40**, **43**, **53**-**60**, **63**-**65**, **67**, **74**-**82**, **85**-**88**, **90**-**94**, **96**-**99**, **101**, **102**, **106**-**108**, **110**-**126**, **134**, **135**, **137**-**139**). Among the studied amine precursors (**13**, **25**, **31**, **35**, **40** and **43**), compound **43**, containing a 4-aminopiperidine moiety attached to the “proximal” piperidine ring in partial structure A (for structure of A see Figure 2.1, Scheme 2.2 or Table 2.1), showed the highest M_2R affinity (pK_i: 9.73) (Table 2.1), and precursor **35**, containing an acylated 4-aminopiperidine moiety instead of the piperidine ring in partial structure A, exhibited the lowest M_2R affinity (pK_i: 5.61). Whereas precursor compound **31** showed no M_2R selectivity compared to the M_1 and M_4 receptor, the other amine precursors (**13**, **25**, **35**, **40** and **43**) displayed a low to moderate M_2R preference over these MR subtypes (Table 2.1).

Table 2.1. M_1-M_5 receptor binding data (pK_i values) of compounds **6**, **13**, **25**, **31**, **35**, **40**, **43**, **53-60**, **63-65**, **73-82** and **134**.



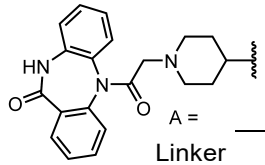
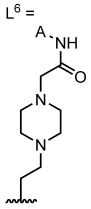
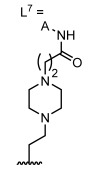
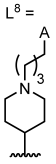
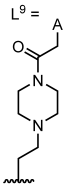
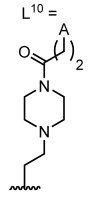
			pK_i[a]					M_2R selectivity $K_i(M_{1,3,4,5}R)/K_i(M_2R)$			
	Linker	Structure	M_1R	M_2R	M_3R	M_4R	M_5R	M_1	M_3	M_4	M_5
6	n.a.	see Figure 2.1	6.49 ± 0.06	8.97 ± 0.05	6.17 ± 0.04	7.04 ± 0.07	7.14 ± 0.05	300	620	84	68
13	n.a.		7.35 ± 0.21	8.45 ± 0.05	-	7.73 ± 0.17	-	16	-	5.9	-
25			6.14 ± 0.09	6.81 ± 0.09	-	5.82 ± 0.06	-	4.6	-	9.6	-
31			5.53 ± 0.10	5.94 ± 0.03	-	5.96 ± 0.08	-	2.4	-	0.87	-
35			4.32 ± 0.09	5.61 ± 0.04	-	4.59 ± 0.01	-	20	-	10	-
40			6.44 ± 0.07	7.63 ± 0.03	-	6.44 ± 0.05		16	-	16	-
43			8.59 ± 0.18	9.73 ± 0.06	-	8.60 ± 0.06	-	16	-	14	-
53		Ac-Arg-Ala-Gly-Arg-NH-L^1	8.19 ± 0.05	8.64 ± 0.06	-	7.80 ± 0.13	-	2.8	-	7.2	-
54		Ac-Arg-Ala-Arg-NH-L^1	7.67 ± 0.11	8.14 ± 0.04	-	7.70 ± 0.04	-	3.1	-	2.8	-
55		Ac-Arg-Ala-Gly-NH-L^1	7.29 ± 0.06	7.83 ± 0.10	-	7.42 ± 0.16	-	3.3	-	2.7	-
56	L^1 = 	Ac-Arg-Ala-D-Arg-NH-L^1	7.28 ± 0.03	7.88 ± 0.10	-	6.45 ± 0.04	-	3.8	-	26	-
57		Ac-Arg-Ala-NH-L^1	7.33 ± 0.09	8.61 ± 0.08	-	7.61 ± 0.07	-	19	.	10	-
58		Ac-Orn-Arg-NH-L^1	7.17 ± 0.07	8.53 ± 0.01	-	6.85 ± 0.05	-	24	-	49	-
59		Ac-D-Orn-Arg-NH-L^1	7.48 ± 0.06	8.09 ± 0.08	-	6.85 ± 0.03	-	4.0	-	17	-
60		Ac-Orn-Agb-NH-L^1	7.41 ± 0.09	8.72 ± 0.11	-	7.16 ± 0.01	-	20	-	35	-
63	L^2 = 	Ac-Lys-Arg-NH-L^2	5.72 ± 0.01	7.13 ± 0.26	<5	5.98 ±	<5	27	>160	16	>170
64		Ac-Orn-Arg-NH-L^2	6.12 ± 0.05	7.34 ± 0.04	-	5.83 ± 0.06	-	17	-	33	-
65		Ac-D-Orn-Arg-NH-L^2	5.84 ± 0.24	6.72 ± 0.11	-	5.67 ± 0.02	-	7.3	-	11	-
73	L^3 = 	H-D-Arg-NH-L^3	9.16 ± 0.16	9.64 ± 0.05	-	8.74 ± 0.18	-	3.4	-	9.2	-
74		H-L-Arg-NH-L^3	8.87 ± 0.05	9.99 ± 0.09	-	9.10 ± 0.09	-	12	-	7.5	-
75		Ac-Arg-Ala-Arg-NH-L^3	9.32 ± 0.08	9.72 ± 0.04	-	9.18 ± 0.05	-	2.6	-	3.4	-
76		Ac-Arg-Ala-Gly-Arg-NH-L^3	9.04 ± 0.05	9.68 ± 0.07	-	9.14 ± 0.13	-	4.3	-	3.6	-

- Table 2.1 continued -

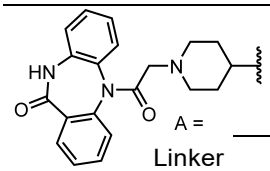


Linker	Structure	pK_i[a]					M_2R selectivity			
							$K_i(M_{1,3,4,5}R)/K_i(M_2R)$			
		M_1R	M_2R	M_3R	M_4R	M_5R	M_1	M_3	M_4	M_5
77	Ac-Arg-Ala-NH-L[3]	9.14 ± 0.14	9.62 ± 0.04	-	8.84 ± 0.03	-	3.3	-	5.9	-
78	Ac-Arg-Ala-Gly-NH- L[3]	9.07 ± 0.05	9.51 ± 0.09	-	9.13 ± 0.20	-	2.6	-	2.7	-
79	Ac-Orn-Ala-Arg-NH- L[3]	8.82 ± 0.10	9.98 ± 0.06	-	8.42 ± 0.01	-	15	-	36	-
80	Ac-D-Orn-Ala-Arg-NH- L[3]	9.02 ± 0.11	9.71 ± 0.14	-	8.84 ± 0.19	-	4.7	-	7.9	-
81	Ac-Orn-Ala-Agb-NH- L[3]	8.90 ± 0.03	10.1 ± 0.06	-	8.48 ± 0.05	-	14	-	38	-
82	Ac-Asn-Agb-Orn-NH- L[3]	8.90 ± 0.15	9.75 ± 0.12	-	8.50 ± 0.09	-	6.7	-	18	-
85	Ac-L-Arg-NH-L[4]	7.93 ± 0.04	9.23 ± 0.03	6.60 ± 0.11-	8.40 ± 0.14	6.17 ± 0.05	20	460	7.5	1300
86	Ac-D-Arg-NH-L[4]	8.11 ± 0.03	9.36 ± 0.06	-	8.37 ± 0.08	-	17	-	10	-
87 (UR-CG176)	Ac-Lys-Ala-Arg-NH-L[4]	7.72 ± 0.08	9.48 ± 0.08	5.92 ± 0.07	7.96 ± 0.03	6.28 ± 0.01	58	3600	33	1500
88	Ac-Lys-Arg-NH-L[4]	8.42 ± 0.06	9.67 ± 0.07	6.76 ± 0.11	8.51 ± 0.06	6.65 ± 0.03	17	840	14	1000
89 (UR-CG188)	Ac-Orn-Ala-Arg-NH-L[4]	7.53 ± 0.02	9.60 ± 0.06	5.88 ± 0.08	7.87 ± 0.09	6.22 ± 0.04	110	5200	55	2300
90	Ac-D-Orn-Ala-Arg-NH-L[4]	8.04 ± 0.09	9.59 ± 0.01	-	8.12 ± 0.04		37	-	30	-
91 (UR-CG205)	Ac-Orn-Ala-Agb-NH-L[4]	7.79 ± 0.10	9.50 ± 0.05	5.94 ± 0.05	7.91 ± 0.04	6.06 ± 0.05	57	3600	38	2700
92 (UR-CG260)	Ac-Asn-Agb-Orn-NH-L[4]	8.09 ± 0.05	9.73 ± 0.01	6.19 ± 0.06	8.11 ± 0.03	6.21 ± 0.02	44	3500	41	3300
93	Ac-Orn-Ala-D-Arg-NH-L[4]	8.17 ± 0.08	9.42 ± 0.01	6.12 ± 0.12	7.98 ± 0.09	5.95 ± 0.02	18	2100	29	2700
94	Ac-Arg-Ala-Gly-Arg-NH-A	4.90 ± 0.04	5.88 ± 0.03	-	4.70 ± 0.15	-	9.5	-	17	-
95	Ac-Arg-Ala-Arg-NH-A	5.54 ± 0.05	5.15 ± 0.02	<5	<5	<5	0.41	>1.9	>1.5	>1.6
96	Ac-Arg-Ala-Gly-NH-L[5]	4.93 ± 0.03	5.36 ± 0.01	<5	4.99 ± 0.01	<5	2.7	>3.0	2.3	>2.8
97	Ac-Arg-Ala-NH-L[5]	5.16 ± 0.08	5.86 ± 0.07	-	5.15 ± 0.08	-	4.8	-	4.9	-
98	Ac-Arg-Ala-Arg-NH-L[5]	5.40 ± 0.12	6.60 ± 0.05	-	5.96 ± 0.14	-	16	-	4.5	-
99	Ac-Arg-Ala-D-Arg-NH-L[5]	4.99 ± 0.10	5.88 ± 0.21	-	5.03 ± 0.05	-	6.1	-	5.3	-

- Table 2.1 continued -

 A = Linker	Structure	pK_i[a]					M_2R selectivity $K_i(M_{1,3,4,5}R)/K_i(M_2R)$			
		M_1R	M_2R	M_3R	M_4R	M_5R	M_1	M_3	M_4	M_5
101 (UR-CG096)	Ac-Lys-Ala-Arg-NH-L[6]	5.27 ± 0.04	7.05 ± 0.01	<5	5.37 ± 0.08	5.04 ± 0.04	62	>130	51	120
102 	Ac-Lys-Arg-NH-L[6]	6.81 ± 0.07	7.67 ± 0.02	<5	6.51 ± 0.02	<5	7.5	>630	14	>520
103 (UR-CG191)	Ac-Orn-Ala-Arg-NH-L[6]	5.22 ± 0.07	7.20 ± 0.12	<5	5.49 ± 0.04	<5	90	>160	47	>160
104 (UR-CG197)	Ac-Orn-Ala-Agb-NH-L[6]	5.09 ± 0.03	7.09 ± 0.02	<5	5.34 ± 0.08	<5	100	>140	58	>140
105 (UR-CG276)	Ac-Orn-Ala-D-Arg-NH-L[6]	<5	7.12 ± 0.08	<5	5.33 ± 0.05	<5	>140	>160	59	>140
106	Ac-Orn-Ala-D-Agb-NH-L[6]	5.70 ± 0.05	7.36 ± 0.01	-	5.90 ± 0.02	-	47	-	29	-
107 (UR-CG220) 	Ac-Lys-Ala-Arg-NH-L[7]	7.22 ± 0.04	9.03 ± 0.03	4.98 ± 0.03	7.39 ± 0.02	5.24 ± 0.03	66	11000	44	6300
108 (UR-CG221)	Ac-Orn-Ala-Agb-NH-L[7]	7.21 ± 0.09	8.93 ± 0.13	5.02 ± 0.05	7.23 ± 0.06	5.29 ± 0.05	50	7600	47	4000
109 (UR-CG239)	Ac-Orn-Ala-Arg-NH-L[7]	7.27 ± 0.12	9.08 ± 0.03	5.07 ± 0.16	7.40 ± 0.03	5.31 ± 0.07	70	11000	48	6000
110	H-L-Arg-NH-L[8]	8.41 ± 0.02	9.53 ± 0.07	-	8.54± 0.07	-	13	-	10	-
111 	H-D-Arg-NH-L[8]	9.03 ± 0.11	9.85 ± 0.10	-	8.69 ± 0.02	-	6.7	-	14	-
112	Ac-Lys-Ala-Arg-NH-L[8]	8.94 ± 0.17	9.54 ± 0.04	-	8.39 ± 0.02	-	4.7	-	14	-
113	Ac-Orn-Ala-Arg-NH-L[8]	8.70 ± 0.06	9.69 ± 0.14	-	8.46 ± 0.02	-	7.1	-	12	-
114	Ac-Orn-Ala-Agb-NH-L[8]	8.89 ± 0.13	9.82 ± 0.10	-	8.57 ± 0.11	-	8.6	-	18	-
115 	Ac-Arg-Ala-Gly-NH-L[9]	5.76 ± 0.06	6.98 ± 0.06	-	5.71 ± 0.13	-	16	-	19	-
116	Ac-Lys-Ala-Arg-NH-L[9]	5.21 ± 0.13	6.61 ± 0.02	<5	5.20 ± 0.05	5.06 ± 0.01	28	>55	26	35
117	Ac-Orn-Ala-Arg-NH-L[9]	5.10 ± 0.12	6.49 ± 0.05	-	5.29 ± 0.10	-	26	-	16	-
118	Ac-Orn-Ala-Agb-NH-L[9]	5.42 ± 0.02	6.49 ± 0.05	-	5.02 ± 0.03	-	12	-	29	-
119 	Ac-Lys-Ala-Arg-NH-L[10]	6.08 ± 0.09	7.39 ± 0.10	<5	5.53 ± 0.04	<5	20	>260	68	>240
120	Ac-Orn-Ala-Arg-NH-L[10]	6.29 ± 0.15	6.84 ± 0.06	<5	5.60 ± 0.06	5.32 ± 0.06	4.0	>91	17	33
121	Ac-Orn-Ala-Agb-NH-L[10]	6.27 ± 0.13	7.28 ± 0.05	<5	5.53 ± 0.06	<5	11	>240	56	>210

- Table 2.1 continued -

 A = Linker	Structure	pK_i[a]					M_2R selectivity $K_i(M_{1,3,4,5}R)/K_i(M_2R)$			
		M_1R	M_2R	M_3R	M_4R	M_5R	M_1	M_3	M_4	M_5
122	Ac-Lys-Ala-Arg-NH-L^{11}	6.55 ± 0.07	7.92 ± 0.07	5.03 ± 0.06	6.03 ± 0.05	5.10 ± 0.05	24	770	77	670
123	Ac-Lys-Arg-NH-L^{11}	6.65 ± 0.08	7.75 ± 0.12	-	6.23 ± 0.05	-	12	-	31	-
124	Ac-Orn-Ala-Arg-NH-L^{11}	6.85 ± 0.06	7.71 ± 0.06	-	6.15 ± 0.07	-	7.2	-	36	-
125	Ac-D-Orn-Ala-Arg-NH-L^{11}	6.59 ± 0.06	7.34 ± 0.04	<5	6.14 ± 0.04	5.58 ± 0.06	5.7	>290	16	59
126	Ac-Orn-Ala-Agb-NH-L^{11}	7.06 ± 0.15	7.77 ± 0.02	-	6.20 ± 0.05	-	5.7	-	37	-
134	H-Asp-Agb-Orn-NH-L^3	8.94 ± 0.05	9.67 ± 0.03	-	8.41 ± 0.07	-	5.7	-	19	-
135	H-Asp-Agb-Orn-NH-L^4	8.15 ± 0.09	9.45 ± 0.01	-	8.36 ± 0.08	-	21	-	13	-
137	Ac-Lys-Ala-Arg-NH-L^{12}	-	5.21 ± 0.08	-	-	-	-	-	-	-
138	Ac-Orn-Ala-Arg-NH-L^{12}	-	5.19 ± 0.09	-	-	-	-	-	-	-
139	Ac-Orn-Ala-Agb-NH-L^{12}	-	5.35 ± 0.05	-	-	-	-	-	-	-

[a]Determined by competition binding with [^{3}H]NMS (K_d values/applied concentrations: M_1, 0.17/0.2 nM; M_2, 0.10/0.2 nM; M_3, 0.12/0.2 nM; M_4, 0.052/0.1 nM; M_5, 0.20/0.3 nM) at live CHO-hM_xR cells (x = 1-5) at 23 °C. Means ± SEM from two (**63**, M_1R, M_5R) or at least three independent experiments (each performed in triplicate); n.a., not applicable.

The subset of compounds derived from the previously described amine precursor **52**[30] (**53-60**) exhibited comparable M_2R affinities (pK_i: 7.83-8.72) (Table 2.1). The highest M_2R selectivities within this subset of compounds were observed for **58** (K_i $M_1R/M_2R/M_4R$ = 24:1:49) and **60** (K_i $M_1R/M_2R/M_4R$ = 20:1:35), containing the peptide moieties Ac-Orn-Arg- and Ac-Orn-Agb-, respectively. DIBA-peptide conjugates derived from the previously described amine precursor **62**[30] (**63-65**) showed moderate M_2R affinities with pK_i values of 6.72−7.34 (Table 1). Among these compounds, **63** exhibited the highest M_2R selectivity (K_i $M_1R/M_2R/M_3R/M_4R/M_5R$ = 27:1:>160:16:>170). All compounds derived from the reported piperazine-containing precursor **72**[33] (**73-82**) showed high M_2R affinities with pK_i values > 9.5 (Table 2.1). This subgroup of DIBA-peptide conjugates comprised comparatively large variations of the peptide moiety (only one amino acid up to tetrapeptides, D-configured amino acids). However, most of these compounds exhibited a low preference for the M_2R. Only compounds **79** and **81** showed a more than 10-fold preference for the M_2R over both the M_1 and M_4 receptor (K_i $M_1R/M_2R/M_4R$ = 15:1:36 and 14:1:38, respectively). Worth mentioning, compound **75**, being structurally identical to the recently reported highly M_2R

selective ligand **4** (cf. Figure 2.1) except for the N-terminal amino acid (Arg in **75** vs. Lys in **4**), displayed, in contrast to **4**, no M_2R selectivity over the M_1 and M_4 receptor. This demonstrated that the type of basic amino acid in the peptide moiety can have high impact on M_2R selectivity.

The DIBA-peptide conjugates **85-93**, derived from the shorter homologue of **72**, i.e. amine precursor **13**, showed also high M_2R affinities (pK_i: 9.23-9.73). Several members of this subset of compounds (**87**, **89**, **91** and **92**) displayed pronounced M_2R selectivity over the M_1R and M_4R, and excellent M_2R selectivity over the M_3 and M_5 receptor. Compound **89** (UR-CG188), containing the peptide moiety Ac-Orn-Ala-Arg-, exhibited the most pronounced M_2R selectivity (K_i $M_1R/M_2R/M_3R/M_4R/M_5R$ = 110:1:5200:55:2300) (Table 2.1). Compounds **87**, **89** and **91** are also closely related to the recently reported DIBA-peptide conjugate **4** (cf. Figure 2.1), which was prepared from amine precursor **72**, the higher homologue of precursor **13**. Interestingly, compound **92**, containing, compared to **87**, **89** and **91**, a markedly different peptide moiety (Ac-Asn-Agb-Orn-), showed a MR selectivity profile comparable to that of **91** (Table 2.1). Compounds **94**-**99**, containing no (**94**, **95**) or a very short (**96**-**99**) linker, showed low M_2R affinities (pK_i: 5.15-6.60) and a low preference for the M_2R compared to the M_1 and M_4 receptor. The subset of compounds derived from precursor **35** (**101-106**), containing an amide group and a bisalkylated piperazine ring, exhibited moderate M_2R affinity (pK_i: 7.05-7.67), but well pronounced M_2R selectivity (except for **102** containing no Ala in contrast to **101** and **103**-**106**) (Table 2.1). The DIBA-peptide conjugates **107**-**109**, which were derived from amine precursor **40**, representing the higher homologue of precursor **35**, showed higher M_2R affinities (pK_i: 8.93-9.08) compared to compounds derived from **35**, and displayed also high M_2R selectivity (e.g. **109**: K_i $M_1R/M_2R/M_3R/M_4R/M_5R$ = 70:1:11,000:48:6000). As compounds **107**-**109** represent the higher homologues of **101**, **104** and **103**, respectively (identical peptide moieties, linker length elongated by one methylene group), these data demonstrated that this type of linker is favorable for high M_2R selectivity and the length of the linker is critical with respect to MR affinity (Table 2.1). Compounds **110**-**114**, derived from precursor **43**, containing an N-alkylated piperidine ring in addition to the N-alkylated piperidine ring of substructure A, showed high M_2R affinities (pK_i: 9.53-9.85), but low M_2R selectivity over the M_1 and M_4 receptor (Table 2.1). The DIBA-peptide conjugates **115**-**126**, which were prepared from the homologues amine precursors **23** (used for **115**-**118**), **24** (used for **119-121**) and **25** (used for **122**-**126**), containing an acylated piperazine ring and varying in length (one (**23**), two (**24**) or three (**25**) methylene groups in between the acylated piperazine ring and the piperidine ring), showed moderate to high M_2R affinity (pK_i: 6.49-7.92).

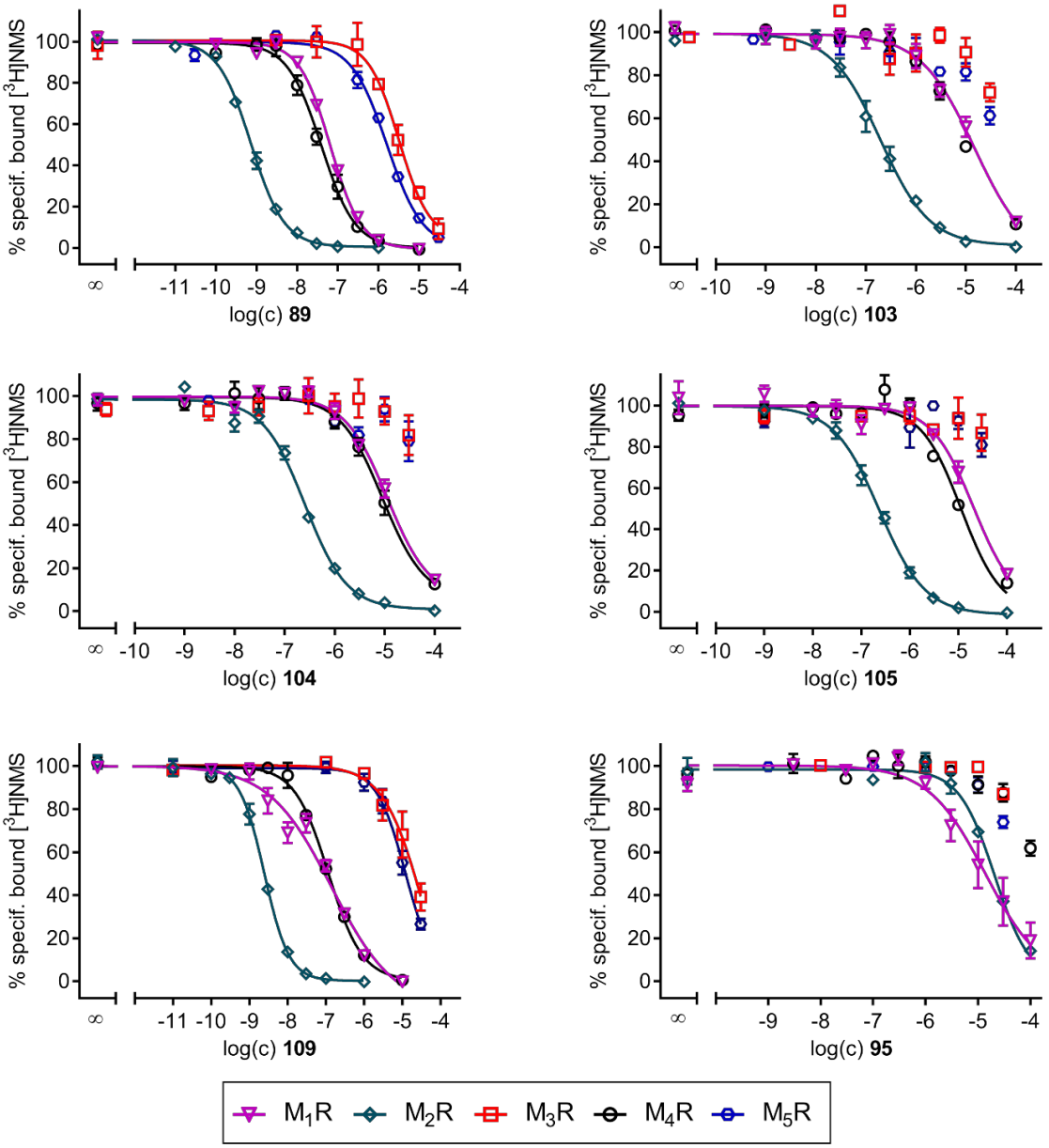


Figure 2.3. Radioligand displacement curves obtained from competition binding experiments with [^{3}H]NMS (0.2 nM (M_1R, M_2R, M_3R), 0.1 nM (M_4R) or 0.3 nM (M_5R) and compounds **89**, **103-105**, **109** and **95** at intact CHO hM_xR cells (x = 1-5). M_3R and M_5R data of **95**, **103-105** and M_4R data of **95** could not be fitted. Data present mean values ± SEM from at least three independent experiments (each performed in triplicate).

M_2R binding increased with linker length and **115-126** showed low or moderate M_2R selectivity over the subtypes M_1 and M_4 (Table 2.1). Compounds **134** and **135**, containing a cyclic peptide moiety, showed high M_2R affinities, but poor M_2R selectivity. Worth mentioning, the linear counterpart of **135**, compound **92**, showed high M_2R affinity and high M_2R selectivity (K_i M_1R/M_2R/M_3R/M_4R/M_5R = 44:1:3500:41:3300) (Table 2.1). In order to demonstrate that M_2R binding of the presented DIBA-peptide conjugates is mainly mediated by the DIBA pharmacophore, the peptides **137-139**, which are present in the M_2R selective

ligands **68**, **70** and **83**, were studied with respect to M_2R binding. When used at high concentrations, peptides **137**-**139** were capable of displacing [^{3}H]NMS from the M_2R (pK_i: 5.19-5.35) (Table 2.1). It should be noted that the interactions of **137**-**139** with the M_2R are most likely different from the interactions of these peptide moieties with the receptor when coupled to the dibenzodiazepinone pharmacophore.

The investigation of the previously reported highly M_2R selective diaryl sulfone **6**[36],the most M_2R selective MR ligand reported to date, with respect to M_1R-M_5R binding in our laboratory yielded a lower M_2R selectivity compared to the reported data (K_i $M_1R/M_2R/M_3R/M_4R/M_5R$ = 300:1:620:84:68 (determined in this study) vs. 2900:1:2700:150:190[36]), showing that **6** is only slightly superior to, for instance **89**, with respect to M_2 over M_1 receptor and M_2 over M_4 receptor selectivity, and inferior to **89** in terms of M_2 over M_3 receptor and M_2 over M_5 receptor selectivity.

2.2.3. Mini-G_{si} protein recruitment assay

The DIBA-peptide conjugates **89**, **103** and **104**, showing high M_2R selectivity, were investigated in a mini-G_{si} protein recruitment assay at HEK-hM_2R cells to study their antagonistic activity at the M_2 receptor. The assay, which is based on the split nanoluciferase technique, was set up according to a protocol recently used for the establishment of mini-G_{si} protein recruitment assays for histamine receptors.[38] First, the MR agonist iperoxo was studied in agonist mode to obtain concentration response curves (CRCs) (see control curves in Figure 2.4B). Subsequently, the inhibiting effect of antagonists **89**, **103** and **104** on the response elicited by 100 nM iperoxo, approximately corresponding to the EC_{80} of iperoxo, was determined (Figure 2.4A, resulting pK_b values see Table 2.2). Additionally, CRCs of iperoxo in the presence of a fixed concentration of the antagonists **89** or **103** (**89**: 58 nM, **103**: 16 µM) were acquired, resulting in a parallel rightward shift of the CRC of iperoxo by approximately two log units (Figure 2.4B). Based on these data, pK_b values were calculated according to the Gaddum

Table 2.2. M_2R binding data (pK_i and pK_b values) of atropine, **89**, **103** and **104** obtained from radioligand competition binding and functional studies.

	pK_i[a]	pK_b[b]	pK_b[c]
atropine	9.04 ± 0.08	8.95 ± 0.04	-
89	9.60 ± 0.06	9.57 ± 0.08	9.06 ± 0.11
103	7.20 ± 0.12	7.13 ± 0.09	7.00 ± 0.08
104	7.09 ± 0.02	6.93 ± 0.05	-

[a]Data taken from Table 2.1 (**89**, **103**, **104**) or from ref.[32] (atropine). [b]Determined in a mini G_{si}-recruitment assay at HEK293T NlucN-mini-G_{si}/hM_2R-NlucC cells at 37°C by inhibition of the effect elicited by 100 nM iperoxo. [c]Determined in a mini Gα_i-recruitment assay at HEK293T NlucN-mini-G_{si}/hM_2R-NlucC cells at 37°C by constructing CRCs with iperoxo in the absence and in the presence of **89** (58 nM) or **103** (16 µM) and calculation of the pK_b from the shift of the EC_{50} of iperoxo (Gaddum equation). Presented are means ± SEM from at least five independent experiments (each performed in triplicate).

equation[39] (Table 2.2). The pK_b values obtained for **89**, **103** and **104** by inhibition of the response elicited by 100 nM iperoxo (Figure 2.4A) were in excellent agreement with their pK_i values determined in the radioligand competition binding assay (Table 2.2). Likewise, the pK_b values of **89** and **103**, derived from the rightward-shifted CRC of iperoxo (Figure 2.4B), were in good accordance with the pK_i values. Representative time-courses of the bioluminescence signals obtained from the mini-G_{si} recruitment assays are shown in Figure S10 (2.5 Supplementary Information).

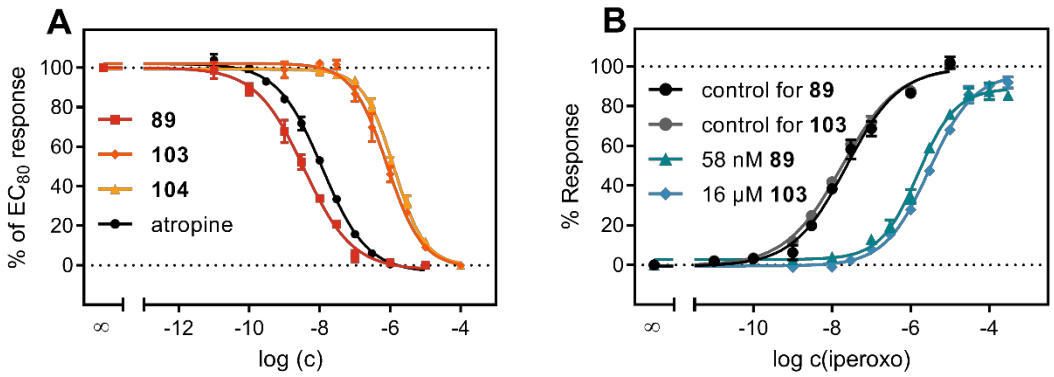


Figure 2.4. M_2R antagonism of compounds **89**, **103** and **104** investigated in a mini-G_{si} protein recruitment assay using HEK293T NlucN-mini-G_{si}/hM_2R-NlucC cells. (A) Concentration-dependent inhibition of the mini-$G\alpha_i$ recruitment to the M_2R elicited by the superagonist iperoxo (100 nM, equals EC_{80}) by the MR antagonists atropine (structure see Figure S10, 2.5. Supplementary Information), **89**, **103** and **104**; for pK_b-values see Table 2.2. (B) Effects of **89** (58 nM) and **103** (16 µM) on the concentration-response-curve (CRC) of iperoxo. The concentrations of **89** and **103** corresponded to 20-fold their IC_{50} obtained from experimental data shown in (A). The measuring periods were 45 min (**103**) or 90 min (**89**) upon addition of iperoxo. In the presence of **89** and **103**, the CRC of iperoxo was rightward shifted (for pK_b-values see Table 2.2). The pEC_{50} values of iperoxo were 7.68 ± 0.044 (mean ± SEM, n = 5) (45 min) and 7.63 ± 0.074 (mean ± SEM, n = 5) (90 min). Data represent mean values ± SEM from at least three independent experiments (each performed in triplicate).

2.2.4. Induced-fit docking

The design of the DIBA-peptide conjugates, presented in this work, was supported by induced-fit docking studies using the respective tool implemented in Schrödinger LLC. Among the prepared M_2R ligands, compounds **60**, **73**, **74**, **81**, **85-87**, **94**, **96-99**, **101**, **110**, **111**, **116**, **119**, **122** and **135** had been docked to the hM_1R (PDB-ID: 5CXV[40]), hM_2R (PDB-ID: 3UON[41]) and hM_4R (PDB-ID: 5DSG[40]) before they were synthesized. Docking to the M_3 and M_5 receptor was not performed as the vast majority of reported dibenzodiazepinone-type MR ligands exhibit high M_2R selectivity compared to these two subtypes. For assessment of the docking results by visual inspection, binding poses corresponding to lowest or second lowest XP GlideScores, were used (low score values are supposed to correlate with a high free energy of binding and thus with high binding affinity). It should be noted that, for a given compound, the predictive value of the XP GlideScores with respect

to M_2R over M_1R and M_2R over M_4R selectivity was low. i.e. the score ratios did not correlate with M_2 over M_1 and M_2 over M_4 receptor selectivity. In addition to the aforementioned compounds, which were synthesized and pharmacologically characterized, further 118 virtual DIBA-peptide conjugates were studied by induced-fit docking to the hM_1, hM_2 and hM_4 receptor. The SMILES strings of these compounds, which were not synthesized, are listed in Table S1 (2.5. Supplementary Information).

The DIBA peptide conjugates **103** and **109**, differing only in linker length by one methylene group, were studied by induced-fit docking at all MR subtypes after their pharmacological characterization for the following reasons: both compounds exhibited high M_2R selectivity, but **109**, representing the higher homologue of **103**, showed approximately 100-fold higher M_2R affinity (Table 2.1). Docking to the M_2R was performed using the recently reported crystal structure of the M_2R bound to the pyridobenzodiazepinone derivative AF-DX 384 (PDB-ID: 5ZKB[42]). According to the lowest-score docking pose, the piperidinylacetyl-dibenzodiazepinone pharmacophore (in the following referred to as headgroup) was localized within the orthosteric binding pocket below the tyrosine lid with nearly identical headgroup orientations for both ligands (Figure 2.5, upper panel). Consequently, the peptide moieties were oriented towards the receptor surface (Figure 2.5B and 2.5C). When comparing the linkers in **103** and **109**, the shorter homologue (**103**) showed rather a stretched conformation, whereas the more flexible linker in **109** was markedly bent (Figure 2.5B and 2.5C, 2.5. Supplementary Figure S12), most likely influencing the orientation of the peptide moieties, which appeared to be different (Figure 2.5, lower panel, 2.5. Supplementary Figure S12). This was consistent with the experimental data, which revealed that the length and flexibility of the linkers in **103** and **109** are critical with respect to M_2R binding (100-fold lower M_2R affinity of **103** compared to **109**). Besides various polar interactions (H-bonding) between the headgroup of the ligands with the receptor (Figure 2.5A and 2.5D), the distances between the tyr residues of the tyrosine lid and the piperidine ring of the ligand headgroup were compatible with π-cation interactions (not indicated in Figure 2.5). Additionally, also π-cation interactions between the tyrosine lid and the piperazine moieties of the ligands might contribute to receptor binding.

According to the induced-fit docking results, both **103** and **109** address two Glu residues in the extracellular loop 2 (ECL2) of the M_2R via basic amino acids of their peptide moieties (**103**: Arg, **109**: Arg and Orn; Figure 2.5E and 2.5H). Interestingly, these two Glu residues are only present in the ECL2 of the M_2R (2.5. Supplementary Figure S13). This indicated that the high M_2R selectivity of dualsterically binding DIBA-peptide conjugates such as **103** and **109** can be attributed, in part, to differences in the occurrence of acidic and basic amino

acids at the surface of MRs (2.5. Supplementary Figures S13 and S14). It should be noted that the head group of **103** and **109** was also located in the orthosteric binding site of the M_1R and M_3R-M_5R according to "low-score" induced-fit docking results obtained for these MR subtypes (2.5. Supplementary Figure S14).

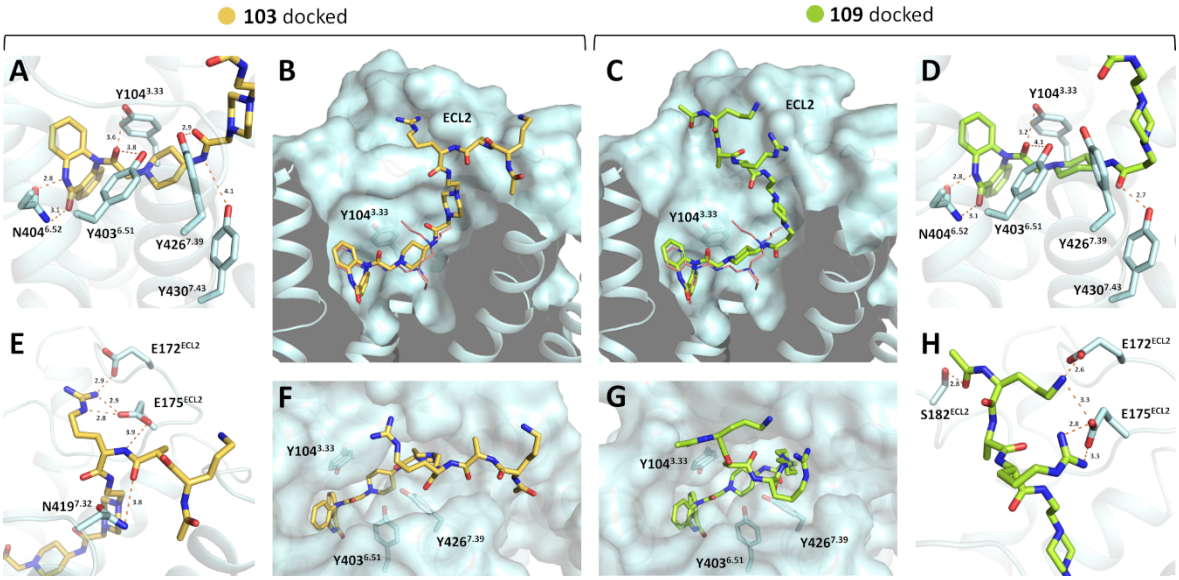


Figure 2.5. Docking poses of **103** (**A**, **B**, **E**, **F**) and **109** (**C**, **D**, **G**, **H**) in the M_2 inactive X-Ray structure (PDB-ID 5ZKB) obtained by induced-fit docking. The lowest scored (best ranked) docking poses are shown. The upper panel shows the orientation and polar interactions of the headgroup (piperidinylacetyl-dibenzodiazepinone moiety) in the orthosteric binding pocket (**A**, **D**), and provides a comparison of the ligand docking poses with the coordinates of AF-DX 384 (red sticks, structure see 2.5. Supplementary Figure S15) from the X-Ray structure (**B**, **C**). The lower panel shows the polar interactions observed between the terminal tripeptide of the ligands and amino acids located in ECL2 of the M_2R (**E**, **H**), and contains a top view on the receptor vestibule (**F**, **G**). The orientation of the Tyr residues of the tyrosin lid (Tyr104, Tyr403, Tyr426) and its proximity to the ligands is visualized in **A**, **F**, **G**, and **D**. Distances, indicated in **A**, **D**, **E** and **H**, are given in Angström.

2.3. Conclusion

Conjugation of short peptides, containing at least one basic amino acid, to dibenzodiazepinone-type MR antagonists via different linkers gave DIBA-peptide conjugates with low to high M_2R affinity and low to high M_2R selectivity. It was demonstrated, that the type and the length of the linker strongly determines M_2R selectivity, a question, which was not addressed in the previous study.[34] Moreover, the introduction of, e.g. D-configured amino acids or homologues of proteinogenic amino acids (Orn vs. Lys, Agb vs. Arg) had rather little impact on M_2R selectivity. As all highly M_2R selective ligands, identified in this study, contain a basic piperazine moiety, putatively interacting with aromatic amino acid residues of the M_2R (cation-π interactions), the incorporation of a basic group, such as a piperazine ring, in the right distance to the tricyclic dibenzodiazepinone core, is advantageous in terms of high M_2R affinity, and, in combination with an appropriate basic

peptide, also with respect to high M_2R selectivity. The presented M_2R selective ligands might serve as lead structures to develop new non-peptidic highly selective M_2R ligands with reduced basicity. Furthermore, the present study can support the design of labeled tool compounds such as radiolabeled and fluorescent M_2R ligands with high M_2R selectivity.

2.4. Experimental section

2.4.1. General experimental conditions

Chemicals for synthesis were purchased from Acros Organics (Geel, Belgium), Iris Biotech (Marktredwitz, Germany), Alfa Aesar (Karlsruhe, Germany), Merck (Darmstadt, Germany), Sigma-Aldrich (Taufkirchen, Germany), Carbolution (St. Ingbert, Germany), or TCI Europe (Zwijndrecht, Belgium). Compound **7** was purchased from Ark Pharm (Arlington Heights, USA), compound **14** and **26** were purchased from abcr (Karlsruhe, Germany), compound **15** was obtained from Enamine (Kiev, Ukraine), compound **16** was purchased from Sigma Aldrich and compound **36** was obtained from Combi-Blocks (San Diego, USA).

Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma-Aldrich and Leibovitz' L-15 medium (L-15) from Fisher Scientific (Nidderau, Germany). FBS, trypsin/EDTA and geneticin (G418) were from Merck Biochrom (Darmstadt, Germany). Puromycin was from InvivoGen (Toulouse, France), NlucC, NlucN and furimazine were obtained from Promega (Mannheim, Germany). The pcDNA3.1 vector was obtained from Thermo Scientific (Nidderau, Germany).

[^{3}H]NMS (specific activity = 80 Ci/mmol, respectively 75 C/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO) via Hartman Analytics (Braunschweig, Germany) or from Novandi Chemistry AB (Södertälje, Sweden).

Acetonitrile for HPLC (gradient grade) was obtained from Merck (Darmstadt, Germany) or SigmaAldrich. DMF, atropine, EDC × HCl, DIPEA, HFIP, iperoxo, Boc-anthranilic acid and TFA were purchased from Sigma Aldrich. CH_2Cl_2, HOBt, DMF for peptide synthesis and NMP for peptide synthesis were obtained from Acros Organics/Fisher Scientific (Nidderau, Germany). HBTU and piperidine were from Iris Biotech. Boc-D-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asn(Trt)-OH, acetic anhydride, and solid supports for SPPS (H-Ala-, H-Arg(Pbf)-, H-Gly- and H-Lys(Boc)-2-ClTrt PS resin) were purchased from Merck. Fmoc-Orn(Boc)-OH and Boc-D -Arg(Pbf)-OH were purchased from Carbolution Chemicals (St. Ingbert, Germany). Fmoc- D -Orn(Boc)-OH, Fmoc-L-Agb(Pbf,Boc)-OH, Fmoc- D -Agb(Pbf,Boc)-OH, Fmoc- D -

Arg(Pbf)-OH, 2-chlorotritylchloride resin (100-200 mesh, 1.60 mmol Cl/g resin) and PyBOP were purchased from Iris Biotech. Boc-L-Arg(Pbf)-OH was purchased from Sigma-Aldrich (Munich, Germany). Deuterated solvents were purchased from Deutero (Kastellaun, Germany).

The syntheses of compounds **52**,[30] **41**,[33] **62**,[30] and **72**[33] were described elsewhere. Compounds **6**,[36] **8**,[37] and **11** [33] were prepared according to described procedures.

If moisture-free conditions were required, reactions were performed in dried glassware under inert atmosphere (argon). Reactions were monitored by TLC using aluminum plates coated with silica gel (Macherey Nagel, pre-coated TLC sheets ALUGRAM® Xtra SIL G/UV_{254}, Düren, Germany). Spots were detected by UV light (254 nm or 366 nm), by staining with a 1% solution of potassium permanganate in 0.1% aq NaOH or using a 0.3% solution of ninhydrine in n-butanol (for amines). Flash chromatography was performed in glass columns on silica gel (Merck silica gel 60, 40-63 µm) or using the flash system Agilent 971-FP Flash purification workstation from Agilent Technologies, equipped with an UV detector (200-400 nm), a FC fraction collector and SuperFlash cartridges (Santa Clara, USA).

Microwave assisted reactions were performed with an Initiator 2.0 synthesizer (Biotage, Uppsala, Sweden). Millipore water was used throughout for the preparation of stock solutions and HPLC eluents. Polypropylene reaction vessels (1.5 or 2 mL) with screw cap (Süd-Laborbedarf, Gauting, Germany) were used for small scale reactions and for the preparation and storage of stock solutions. Nylon membrane filters (0.2 µm, Phenomenex, Aschaffenburg, Germany) were used for filtrations of the injection solutions for preparative HPLC and reactions mixtures. Temperature-controlled reactions in 1.5-mL reaction vessels were performed using a Thermocell Mixing Block MB-102 from Bioer (Hangzhou, China).

NMR spectra were recorded on a Bruker Avance 400 (7.05 T, ^{1}H: 400.3 MHz, ^{13}C: 100.7 MHz) or a Bruker Avance III HD 600 equipped with a cryogenic probe (14.1 T ^{1}H: 600.3 MHz, ^{13}C: 150.9 MHz) (Bruker, Karlsruhe, Germany). Abbreviations for the multiplicities of the signals are s (singlet), d (doublet), t (triplet), m (multiplet) and brs (broad singlet). High-resolution mass spectrometry (HRMS) analysis was performed on an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA) using an ESI source. Except for **30** and **63**, a Prep 150 LC system from Waters (Eschborn, Germany), consisting of a binary gradient module, a 2489 UV/visible detector, and a Waters fraction collector III, was used as preparative HPLC. Compounds **30** and **63** were purified using a preparative HPLC system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. For the purification of compounds **13**, **17**, **19**, **23**-**25**, **27**, **28**, **30**, **33**-

35, **53-60**, **63**, **73-81**, **85-91**, **94-99**, **101-104**, **110-112**, **115-117**, **119** and **122-125**, a Kinetex-XB C18 (5 µm, 250 mm × 21 mm; Phenomenex, Aschaffenburg, Germany) was used as stationary phase at a flow rate of 20 mL/min, and for the purification of compounds **64**, **65**, **71**, **82**, **92**, **93**, **105-109**, **113**, **114**, **118**, **121**, **126, 128-135** and **137-139** a YMC-Actus Triart C18 (5 µm, 150 × 20 mm, Dinslaken, Germany) served as stationary phase at a flow rate of 20 mL/min.

Mixtures of 0.1% aq TFA and acetonitrile were used as mobile phase. The detection wavelength was set to 220 nm throughout. The solvent of collected fractions was removed by lyophilization using a Scanvac freeze drying apparatus (Labogene, Allerød, Denmark) equipped with a RZ 6 rotary vane vacuum pump (Vacuubrand, Wertheim, Germany). Analytical HPLC analysis was performed with a system from Agilent Technologies composed of a 1290 Infinity binary pump equipped with a degasser, a 1290 Infinity autosampler, a 1290 Infinity thermostated column compartment, a 1260 Infinity diode array detector, and a 1260 Infinity fluorescence detector. A Kinetex-XB C18 (2.6 µm, 100 × 3 mm; Phenomenex) was used as stationary phase at a flow rate of 0.6 mL/min. Mixtures of 0.04% aq TFA (A) and acetonitrile (B) were used as mobile phase. The following linear gradient was applied for compounds **13**, **23-25**, **31**, **35**, **40**, **43**, **53-60**, **63-65**, **73-82**, **85-99**, **101-126, 134** and **135**: 0-12 min, A/B 90:10 to 70:30; 12-16 min, 70:30 to 5:95; 16-20 min, 5:95. For **6** the following linear gradient was applied: 0-12 min, A/B 90:10 to 40:60; 12-16 min, 40:60 to 5:95; 16-20 min, 5:95. For all analytical HPLC runs the oven temperature was set to 25 °C, the injection volume was 20 µL and detection was performed at 220 nm. Compound concentrations of the sample solutions were between 40 and 120 µM. The stock solutions (final concentration: 10 mM) of the final compounds were prepared in DMSO and were stored at -78°C. Retention (capacity) factors were calculated from retention times (t_R) according to $k = (t_R - t_0)/t_0$ (t_0 = dead time).

2.4.2. Compound characterization

Target compounds **6**, **13**, **23-25**, **31**, **35**, **40**, **43**, **53-60**, **63-65**, **73-82**, **85-99**, **101-126**, **134** and **135** were characterized by ^{1}H-NMR, ^{13}C-NMR and 2D-NMR (^{1}H-COSY, HSQC, HMBC) spectroscopy, HRMS and RP-HPLC analysis. HPLC purities amounted to ≥ 96% (220 nm) (chromatograms shown in Appendix).

Annotation concerning the ^{1}H- and ^{13}C-NMR spectra of **53-60**, **63-65**, **73-82**, **85-99**, **101-126**, **134**, **135**: due to a slow rotation about the exocyclic amide group of the

dibenzodiazepinone moiety on the NMR time scale, two isomers (ratios provided in the experimental protocols) were evident in the ^{1}H- and ^{13}C-NMR spectra.

Annotation concerning the ^{1}H-NMR spectra (solvent: DMSO-d_6) of **53-60**, **63-65**, **73-82**, **85-99**, **101-126**, **134**, **135**: in order to integrate signals interfering with the broad water signal at ca 3.5 ppm, additional ^{1}H-NMR spectra were recorded in DMSO-d_6/D_2O (4:1 v/v) (spectra shown in the 2.5. Supplementary Information).

Analytical data of compound **6** are shown in the Supplementary Information.

2.4.3. Solid-phase peptide synthesis (SPPS)

SPPS was performed according to a previously described procedure.[34] Peptides **44-51**, **61**, **68-71**, **83**, **84**, **100** and **127** were used in the next step without purification. The chemical identities and purities of **44-51**, **61**, **68-71**, **83**, **84**, **100** and **127** were assessed by LC-HRMS (data not shown). Peptide **127** was synthesized according to the before mentioned procedure, but without Fmoc-deprotection after the last elongation step. In the case of peptides **47**, **70**, **84** and **100**, the 2-chlorotrityl chloride resin was loaded with the Fmoc-protected C-terminal amino acid of these peptides. For this purpose, the resin was swollen for 1 h at room temperature in CH_2Cl_2. After removing the solvent, the protected amino acid (3-fold excess) and DIPEA (5equiv.) in CH_2Cl_2 was added. The vessel was shaken at 35 °C overnight and washed with CH_2Cl_2 (2 ×). The capping solution (CH_2Cl_2/MeOH/DIPEA 10:3:0.5 v/v/v) was added to the resin and the suspension was shaken for 1 h at room temperature. The resin was washed with CH_2Cl_2 (2 ×) and DMF/NMP (8:2 v/v) (2 ×). After Fmoc-deprotection with 20% piperidine in DMF/NMP (8:2) at room temperature (2 × 10 min), followed by washing with DMF/NMP (8:2) (5 ×), the following coupling steps, required for the synthesis of **47**, **70**, **84** and **100**, were performed according to the reported procedure.[34]

2.4.4. General Procedure for the Synthesis of Compounds 53-60, 63-65, 73-82, 85-99, 101-126, 130, 131, 134, 135, 137-139

The coupling of the side chain protected peptides or amino acids **44-51**, **61**, **66-71**, **83**, **84**, **100** or **127** to amines **13**, **23-25**, **28**, **31**, **35**, **40**, **43**, **52**, **62** or **72** was performed in 1.5 or 2 mL polypropylene reaction vessels with screw cap. HOBt (1.2 equiv.) and EDC× HCl (1.2 equiv.) were added to a cooled (5 °C) solution of the respective side chain-protected peptide (1-1.2 equiv.) in DMF (0.2−0.4 mL). In the case of compounds derived from amine

precursors **13**, **35**, **40** or **72**, a solution of the respective amine precursor (tetrakis(hydrotrifluoroacetate), 1 equiv.) and DIPEA (1.5-2 equiv.) in DMF (0.1 mL) was immediately added and shaking was continued at 5 °C overnight. For the synthesis of compounds derived from amine precursors **23**, **24**, **25** or **43**, a solution of the respective amine precursor (tris(hydrotrifluoroacetate), 1 equiv.) and DIPEA (1.5 equiv.) in DMF (0.1 mL) was immediately added and shaking was continued at 5 °C overnight. In the case of compounds derived from amine precursors **28** or **31**, a solution of the respective amine precursor (bis(hydrotrifluoroacetate), 1 equiv.) and DIPEA (0.5 and 1 equiv., respectively) in DMF (0.1 mL) was immediately added and shaking was continued at 5 °C overnight. For the synthesis of compounds derived from propylamine **136**, a solution of propylamine (3 eq) and DIPEA (0.5 eq) in DMF (0.1 mL) was added and shaking was continued at 5 °C overnight. For the synthesis of compounds derived from **52** or **62** a solution of the respective amine (free base, 1 equiv) in DMF (0.1 mL) was immediately added, and stirring was continued at 5 °C overnight. 1% aq. TFA (19-64 µL, depending on the amount of DIPEA used in the reaction) was added, and the mixture was subjected to preparative HPLC to isolate the protected intermediates. After lyophilisation, these compounds were dissolved in TFA/H_2O (95:5 v/v) and the resulting mixtures were stirred at rt for 3 h. CH_2Cl_2 (ca. 20 mL) was added, and the volatiles were removed by evaporation. This step was repeated once. The final products were purified by preparative HPLC (except for peptides **137**-**139**).

2.4.5. Experimental synthetic protocols and analytical data of compounds 9, 10, 13, 17-19, 23-25, 28, 31, 33, 35, 37, 38, 40, 43, 53-60, 63-65, 73-82, 85-99, 101-126, 130, 131, 134, 135, 137-139

5-(2-(4-(3-Hydroxypropyl)piperidin-1-yl)acetyl)-5,10-dihydro-11*H*-dibenzo[*b*,*e*][1,4] diazepin-11-one (9)

Compound **7** (394 mg, 2.74 mmol) and compound **8** (253 mg, 0.88 mmol) were suspended in acetonitrile (4.5 mL) followed by the addition of freshly grained potassium carbonate (495 mg, 3.53 mmol). The mixture was stirred at 85 °C under microwave irradiation for 90 min and then cooled to room temperature. Remaining solids were separated by filtration and washed with CH_2Cl_2 (2 × 5 mL). The solvent of the combined filtrate and washings was removed under reduced pressure and the residue was dissolved in CH_2Cl_2 (40 mL) followed by washing with water (2 × 40 mL) and brine (40 mL). The organic phase was dried over Na_2SO_4 and the volatiles were removed *in vacuo*. The residue was subjected to flash chromatography (gradient: 0-30 min: CH_2Cl_2/MeOH 100:0 to 90:10) to yield **9** as a pale

brown oil (338 mg, 97%). R_f = 0.4 (CH_2Cl_2/MeOH 9:1 v/v). Ratio of configurational isomers evident in the NMR spectrum: ca 1.5:1. ^{1}H-NMR (400.3 MHz, MeOH-d_4): δ (ppm) 1.06-1.32 (m, 5H), 1.45-1.64 (m, 4H), 1.88-2.00 (m, 2H), 2.46-2.58 (m, 0.4H), 2.59-2.69 (m, 0.6H), 2.82 (t, *J* 11 Hz, 1H), 2.99-3.10 (m, 0.6H), 3.10-3.26 (m, 1.4H), 3.05 (t, *J* 6.8 Hz, 2H), 7.20-7.29 (m, 2H), 7.30-7.43 (m, 1H), 7.43-7.61 (m, 3H), 7.62-7.70 (m, 1H), 7.83-7.94 (m, 1H). ^{13}C-NMR (100.7 MHz, MeOH-d_4): δ (ppm) 29.29, 29.42, 31.54, 32.23, 34.93, 35.58, 53.55, 61.66, 61.77, 121.60, 125.20, 125.54, 127.55, 128.06, 128.49, 129.15, 130.63, 132.88, 134.60, 135.59, 142.50. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{23}H_{28}N_3O_3]^+$: 394.2125, found: 394.2146. $C_{23}H_{27}N_3O_3$ (393.49).

5-(2-(4-(3-Chloropropyl)piperidin-1-yl)acetyl)-5,10-dihydro-11*H*-dibenzo[*b*,*e*][1,4] diazepin-11-one (10)

Alcohol **9** (114 mg, 0.290 mmol) was dissolved in CH_2Cl_2 (1.5 mL) followed by the addition of pyridine (47.0 µL, 0.580 mmol) and thionyl chloride (210 µL, 2.90 mmol). The mixture was refluxed under an atmosphere of argon overnight. After cooling to room temperature, the mixture was quenched by the addition of MeOH. The solvent was removed under reduced pressure and the residue was subjected to flash chromatography (gradient: 0-30 min: CH_2Cl_2/MeOH 100:0 to 90:10) to yield **10** as a pale brown oil (118 mg, 99%). R_f = 0.5 (CH_2Cl_2/MeOH 9:1 v/v). Ratio of configurational isomers evident in the NMR spectrum: ca 1.5:1.^{1}H-NMR (400.3 MHz, MeOH-d_4): δ (ppm) 1.37-1.64 (m, 5H), 1.71-1.83 (m, 2H), 1.92 (t, *J* 14 Hz, 2H), 2.89-3.11 (m, 2H), 3.37-3.46 (m, 2H), 3.56 (t, *J* 6.5 Hz, 2H), 3.62-3.82 (m, 2H), 4.33-4.47 (m, 1H), 7.24-7.32 (m, 0.8 H), 7.32-7.41 (m, 1.6H), 7.44-7.49 (m, 0.6H), 7.49-7.57 (m, 1.6H), 7.59-7.65 (m, 0.4H), 7.65-7.73 (m, 1.6 H), 7.73-7.79 (m, 0.4H), 7.90 (d, *J* 7.9 Hz, 0.6 H), 7.97 (d, *J* 7.9 Hz, 0.4 H). ^{13}C-NMR (100.7 MHz, MeOH-d_4): δ (ppm) 28.86, 29.33, 32.31, 32.40, 44.25, 53.26, 53.58, 56.55, 121.67, 122.24, 125.44, 126.13, 126.57, 127.17, 127.58, 128.13, 128.68, 129.11, 129.52, 129.74, 130.26, 130.57, 130.93, 131.56, 132.14, 133.17, 133.61, 134.06, 134.35, 135.62, 139.76, 141.36, 163.88, 164.29, 167.21, 167.44. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{23}H_{27}ClN_3O_2]^+$: 412.1786, found: 412.1799. $C_{23}H_{26}ClN_3O_2$ (411.93).

5-(2-(4-(3-(4-(2-Aminoethyl)piperazin-1-yl)propyl)piperidin-1-yl)acetyl)-5,10-dihydro-11*H*-dibenzo[*b*,*e*][1,4]diazepin-11-one tetrakis(hydrotrifluoroacetate) (13)

Chloride **10** (425 mg, 1.03 mmol) was suspended in acetone (7 mL). Sodium iodide (1.086 g, 7.24 mmol), amine **11** (273 mg, 1.19 mmol) and potassium carbonate (714 mg, 5.17 mmol) were added and the mixture was refluxed for 72 h. The solvent was evaporated, and the residue was taken up in CH_2Cl_2 (10 mL). Insoluble material was separated by filtration and washed with CH_2Cl_2 (2 × 3 mL). The filtrate and washings were combined, and the solvent was removed under reduced pressure. The residue was subjected to flash chromatography (gradient: 0-25 min: CH_2Cl_2/MeOH 100:0 to 80:20) yielding the Boc-protected intermediate **12** as a pale yellow solid (511 mg, 82%. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{34}H_{49}N_6O_4]^+$: 605.3810, found: 605.3819), which was dissolved in CH_2Cl_2/TFA/H_2O 4:1:0.1 (v/v/v 3 mL). The solution was stirred at room temperature for 3 h followed by evaporation of the volatiles. Purification of the product by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 66:34, t_R = 8 min) yielded compound **13** as a white solid (317 mg, 32%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.20 (s, 2H), 1.33-1-50 (m, 3H), 1.59-1.67 (m, 2H), 1.72-1.86 (m, 2H), 2.35-2.46 (m, 2H), 2.59 (t, *J* 6.2 Hz, 2H), 2.88-3.07 (m, 10H), 3.32-3.38 (m, 1H), 3.40-3.50 (m, 2H), 3.60 (t, *J* 10 Hz, 1H), 3.78 (d, *J* 17 Hz, 0.6H), 3.92 (d, *J* 17 Hz, 0.4H), 4.36-4.50 (m, 1H), 7.22-7.32 (m, 1.6H), 7.32-7.39 (m, 1.2H), 7.40-7.49 (m, 1.2H), 7.50-7.55 (m, 1.4H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.68-7.72 (m, 1 H), 7.72-7.77 (m, 1H), 7.77-7.80 (m, 0.4H), 7.80-7.91 (m, 4.2H), 9.68 (s, 1H), 10.2 (brs, 1H), 10.7 (s, 0.4H), 10.8 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 20.37, 28.48, 31.97, 32.37, 35.58, 48.89, 50.80, 52.48, 52.61, 53.09, 53.13, 55.35, 55.89, 116.60 (q, *J* 297 Hz, TFA), 121.88, 122.31, 124.89, 125.46, 126.76, 127.26, 127.71, 128.26, 128.69, 128.94, 129.55, 129.71, 129.99, 130.38, 130.93, 131.53, 131.63, 133.02, 133.10, 133.76, 134.69, 135.78, 139.46, 140.99, 158.39 (q, *J* 33 Hz, carbonyl group of TFA), 163.68, 164.17, 165.74, 166.03. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{29}H_{41}N_6O_2]^+$: 505.3286, found: 505.3285. RP-HPLC (220 nm): 99% (t_R = 4.3 min, *k* = 4.7). $C_{29}H_{40}N_6O_2 \cdot C_8H_4F_{12}O_8$ (504.68 + 456.06).

2-(1-(2-Oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl) piperidin-4-yl)acetic acid hydrotrifluoroacetate (17)

Compound **14** (354 mg, 2.50 mmol) and compound **8** (205 mg, 0.715 mmol) were dissolved in DMF (4.5 mL) followed by the addition of freshly grained potassium carbonate (523 mg, 3.79 mmol). The mixture was stirred at 85 °C under microwave irradiation for 90 min and

subsequently cooled to room temperature. Insoluble material was separated by filtration and washed with CH_2Cl_2 (2 × 3 mL). The combined filtrate and washings were concentrated under reduced pressure yielding a pale yellow residue. Purification by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 66:34, t_R = 11 min) yielded compound **17** as a pale yellow solid (132 mg, 36%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (400.3 MHz, MeOH-*d*$_4$): δ (ppm) 1.47-1.67 (m, 2H), 1.94-2.07 (m, 3H), 2.23-2.36 (m, 2H), 2.98 (t, *J* 12 Hz, 1H), 3.09 (t, *J* 12 Hz, 1H), 3.40-3.51 (m, 1H), 3.69-3.87 (m, 2H), 4.42 (t, *J* 16 Hz, 1H), 7.23-7.43 (m, 2.4H), 7.46-7.57 (m, 2.2H), 7.60-7.72 (m, 2H), 7.73-7.80 (m, 0.4H), 7.89-7.93 (m, 0.6H), 7.95-8.00 (m, 0.4H). ^{13}C-NMR (100.7 MHz, MeOH-*d*$_4$: δ (ppm) 28.63, 33.66, 36.91, 39.10, 43.69, 49.48, 52.23, 53.38, 53.71, 54.88, 122.25, 126.11, 126.49, 127.08, 127.49, 128.07, 128.71, 129.18, 129.51, 129.87, 120.33, 130.97, 131.64, 133.18, 134.05, 139.60, 167.54, 173.70. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{22}H_{24}N_3O_4]^+$: 394.1761, found: 394.1769. $C_{22}H_{23}N_3O_4 \cdot C_2HF_3O_2$ (393.44 + 114.02).

3-(1-(2-Oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propanoic acid hydrotrifluoroacetate (18)

Compound **18** was prepared from **8** (100 mg, 0.349 mmol) and **15** (207 mg, 1.07 mmol) according to the procedure for the synthesis of **17**. Potassium carbonate: 226 mg, 1.64 mmol. Purification by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 66:34, t_R = 14 min) yielded compound **18** as a pale yellow solid (118 mg, 65%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (400.3 MHz, MeOH-*d*$_4$): δ (ppm) 1.41-1.68 (m, 5H), 1.87-2.02 (m, 2H), 2.34 (t, *J* 7.1 Hz, 2H), 2.93 (t, *J* 11 Hz, 1H), 3.04 (t, *J* 12 Hz, 1H), 3.39-3.51 (m, 1H), 3.69-3.85 (m, 2H), 4.42 (t, *J* 16 HZ, 1H), 7.23-7.43 (m, 2.4H), 7.44-7.58 (m, 2.2H), 7.60-7.79 (m, 2.4H), 7.91 (d, *J* 7.4 Hz, 0.6H), 7.97 (d, *J* 7.7 Hz, 0.4H). ^{13}C-NMR (100.7 MHz, MeOH-*d*$_4$): δ (ppm) 28.82, 30.41, 32.60, 36.14, 53.47, 53.84, 56.66, 119.79, 121.69, 122.25, 125.52, 126.12, 126.47, 127.08, 127.49, 128.05, 128,73, 129.82, 130.32, 130.66, 130.97, 131.63, 132.01, 133.18, 134.05, 134.39, 135.68, 141.30, 167.42, 175.59. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{23}H_{26}N_3O_4]^+$: 408.1918, found: 408.1920. $C_{23}H_{25}N_3O_4 \cdot C_2HF_3O_2$ (407.47 + 114.02).

4-(1-(2-Oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl) piperidin-4-yl)butanoic acid hydrotrifluoroacetate (19)

Compound **19** was prepared from **8** (264 mg, 0.921 mmol) and **16** (574 mg, 2.76 mmol) according to the procedure for the synthesis of **17**. Potassium carbonate: 635 mg, 4.60 mmol. Purification by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 66:34, t_R = 12 min) yielded compound **19** as a white solid (225 mg, 46%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (400.3 MHz, MeOH-d_4): δ (ppm) 1.27-1.38 (m, 2H), 1.39-1.70 (m, 5H), 1.86-2.01 (m, 2H), 2.29 (t, *J* 7.2 Hz, 2H), 2.93 (t, *J* 11 Hz, 1H), 3.04 (t, *J* 11 Hz, 1H), 3.39-3.50 (m, 1H), 3.67-3.85 (m, 2H), 4.41 (t, *J* 16 Hz, 1H), 7.22-7.42 (m, 2.4H), 7.45-7.57 (m, 2.2H), 7.60-7.72 (m, 2H), 7.73-7.79 (m, 0.4H), 7.91 (d, *J* 7.9 Hz, 0.6H), 7.98 (d, *J* 7.4 Hz, 0.4H). ^{13}C-NMR (100.7 MHz, MeOH-d_4): δ (ppm) 21.48, 28.99, 32.89, 33.27, 34.70, 53.58, 53.94, 56.66, 121.69, 122.25, 125.46, 126.12, 126.47, 127.09, 127.49, 128.06, 128.72, 129.16, 129.49, 129.83, 130.65, 130.97, 131.63, 132.03, 133.18, 134.06, 135.67, 139.63, 141.28, 164.05, 167.43, 175.85. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{24}H_{28}N_3O_4]^+$: 422.2074, found: 422.2080. $C_{24}H_{27}N_3O_4 \cdot C_2HF_3O_2$ (421.50 + 114.02).

5-(2-(4-(2-(4-(2-Aminoethyl)piperazin-1-yl)-2-oxoethyl)piperidin-1-yl)acetyl)-5,10-dihydro-11*H*-dibenzo[*b*,*e*][1,4]diazepin-11-one tris(hydrotrifluoroacetate) (23)

The coupling of piperazine derivative **11** to the carboxylic acid **17** was performed in a 2 mL polypropylene reaction vessel with screw cap. HOBt (43.4 mg, 0.283 mmol) and EDC × HCl (54.3 mg, 0.283 mmol) were added to a solution of the acid **17** (92.8 mg, 0.236 mmol) and compound **11** (65.0 mg, 0.283 mmol) in DMF (0.5 mL). DIPEA (100 µL, 0.590 mmol) was immediately added and the solution was shaken at 55 °C overnight. 10% aq TFA (455 µL) was added and the solution was subjected to preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 57:43; t_R = 11 min) to isolate the Boc-protected intermediate **20** as a pale yellow solid (131 mg, 0.157 mmol, 77%. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{33}H_{45}N_6O_5]^+$: 605.3446, found: 605.3455), which was dissolved in TFA/H_2O (95:5 v/v) followed by stirring of the mixture at room temperature for 3 h. CH_2Cl_2 (20 mL) was added and the volatiles were evaporated under reduced pressure. This process was repeated once. The product was purified by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 76:24, t_R = 7 min) yielding amine precursor **23** as a white solid (130 mg, 49%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.37-1.55 (m, 2H), 1.75-1.94 (m, 3H), 2.23-2.36 (m,

2H), 2.79-3.11 (m, 7H), 3.14 (s, 3H), 3.31-3.38 (m, 1H), 3.51-3.79 (m, 5.6H), 3.91 (d, *J* 16.4 Hz, 0.4H), 4.26-4.40 (m, 1H), 7.22-7.31 (m, 1.4H), 7.33-7.38 (m, 1H), 7.42-7.49 (m, 1H), 7.49-7.54 (m, 1.2H), 7.60 (t, *J* 7.6 Hz, 0.4H), 7.69 (t, J 7.7 Hz, 0.6H), 7.72-7.76 (m, 1H), 7.76-7.80 (m, 0.4H), 7.82 (d, *J* 8.3 Hz, 0.6H), 7.89 (d, *J* 7.8 Hz, 0.4H), 8.11 (brs, 3H), 9.70 (brs, 1H), 10.7 (s, 0.4H), 10.8 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 28.63, 29.93, 34.20, 37.56, 42.92, 51.57, 51.84, 52.47, 52.62, 53.10, 55.91, 116.85 (q, *J* 298 Hz, TFA), 119.81, 121.88, 122.34, 124.88, 125.50, 126.77, 127.72, 128.27, 128.70, 128.95, 129.57, 129.72, 130.01, 130.39, 130.94, 131.55, 131.68, 133.03, 133.11, 133.79, 134.70, 135.78, 139.48, 141.01, 158.50 (q, *J* 33 Hz, carbonyl group of TFA), 163.68, 164.18, 165.75, 166.04, 169.19. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{28}H_{37}N_6O_3]^+$: 505.2922, found: 505.2919. RP-HPLC (220 nm): 99% (t_R = 4.2 min, *k* = 4.5). $C_{28}H_{36}N_6O_3 \cdot C_6H_3F_9O_6$ (504.64 + 342.06).

5-(2-(4-(3-(4-(2-Aminoethyl)piperazin-1-yl)-3-oxopropyl)piperidin-1-yl)acetyl)-5,10-dihydro-11*H*-dibenzo[*b*,*e*][1,4]diazepin-11-one tris(hydrotrifluoroacetate) (24)

Compound **24** was prepared from **11** (14.7 mg, 0.0643 mmol) and **18** (21.8 mg, 0.0536 mmol) according to the procedure for the synthesis of **23**. HOBt: 9.80 mg, 0.0643 mmol, EDC × HCl: 12.3 mg, 0.0643 mmol. The Boc-protected intermediate **21** (purified by preparative HPLC; gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 57:43, t_R = 13 min) was obtained as a white solid (24.4 mg, 0.0288 mmol, 54%. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{34}H_{47}N_6O_5]^+$: 619.3602, found: 619.3608). After Boc-deprotection the final amine precursor (**24**) was purified by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 76:24, t_R = 10 min) yielding **24** as a white solid (30 mg, 42%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.29-1.52 (m, 5H), 1.72-1.92 (m, 2H), 2.34 (t, *J* 7.0 Hz, 2H), 2.72-3.07 (m, 7H), 3.13 (s, 3H), 3.27 (d, *J* 11 Hz, 1H), 3.46-3.84 (m, 6H), 4.25-4.37 (m, 1H), 7.22-7.31 (m, 1.4H), 7.32-7.39 (m, 1H), 7.42-7.49 (m, 1H), 7.50-7.55 (m, 1.2H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.68-7.79 (m, 2H), 7.82 (d, *J* 8.1 Hz, 0.6H), 7.88 (d, *J* 7.8 Hz, 0.4H), 8.01 (brs, 3H), 9.58 (brs, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 28.59, 29.04, 30.51, 32.36, 34.23, 42.91, 51.61, 51.91, 52.69, 52.55, 53.16, 55.92, 115.79 (TFA), 117.77 (TFA), 121.88, 122.30, 124.88, 125.47, 126.74, 127.25, 127.70, 128.25, 128.70, 128.25, 128.70, 128.96, 129.56, 129.69, 130.00, 130.37, 130.93, 131.53, 131.61, 133.03, 133.10, 133.76, 134.67, 135.76, 139.44, 140.98, 158.27 (q, *J* 33 Hz, carbonyl group of TFA), 163.70, 164.17, 165.74, 166.03, 170.55. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{29}H_{39}N_6O_3]^+$: 519.3078, found:

519.3085. RP-HPLC (220 nm): 99% (t_R = 4.5 min, k = 4.9). $C_{29}H_{38}N_6O_3 \cdot C_6H_3F_9O_6$ (518.66 + 342.06).

5-(2-(4-(4-(4-(2-Aminoethyl)piperazin-1-yl)-4-oxobutyl)piperidin-1-yl)acetyl)-5,10-dihydro-11*H*-dibenzo[*b*,*e*][1,4]diazepin-11-one tris(hydrotrifluoroacetate) (25)

Compound **25** was prepared from **11** (16 mg, 0.068 mmol) and **19** (24 mg, 0.057 mmol) according to the procedure for the synthesis of **23**. HOBt: 11 mg, 0.068 mmol. EDC × HCl: 13 mg, 0.068 mmol. The Boc-protected intermediate **22** (purified by preparative HPLC; gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 57:43, t_R = 13 min) was obtained as a white solid (26 mg, 0.030 mmol, 52%). After Boc-deprotection the final amine precursor (**25**) was purified by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 76:24, t_R = 9 min) yielding **25** as a white solid (20 mg, 56%. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{35}H_{49}N_6O_5]^+$: 633.3759, found: 633.3763). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.19 (s, 2H), 1.29-1.53 (m, 5H), 1.69-1.87 (m, 2H), 2.30 (t, *J* 7.4 Hz, 2H), 2.75-3.24 (m, 10H), 3.33 (d, *J* 10 Hz, 2H), 3.50-3.93 (m, 5H), 4.36-4.46 (m, 1H), 7.21-7.30 (m, 1.4H), 7.32-7.37 (m, 1H), 7.41-7.48 (m, 1H), 7.48-7.53 (m, 1.2H), 7.58 (t, *J* 7.6 Hz, 0.4H), 7.66-7.70 (m, 0.6H), 7.71-7.79 (m, 1.4H), 7.81 (d, *J* 7.5 Hz, 0.6H), 7.87 (d, *J* 7.9 Hz, 0.4H), 8.12 (brs, 3H), 9.65 (brs, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 21.52, 28.67, 31.93, 32.71, 34.12, 34.92, 42.82, 51.56, 51.86, 52.63, 52.76, 53.09, 53.22, 55.92, 116.88 (q, *J* 298 Hz, TFA), 124.88, 125.48, 126.78, 127.28, 127.73, 128.27, 128.68, 128.93, 129.55, 129.73, 129.99, 130.39, 130.93, 131.53, 131.67, 133.03, 133.13, 133.77, 134.70,.135.78, 139.48, 141.03, 158.49 (q, *J* 33 Hz, carbonyl group of TFA), 163.70, 164.19, 165.75, 166.04, 170.65. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{30}H_{41}N_6O_3]^+$: 533.3235, found: 533.3236. RP-HPLC (220 nm): 99% (t_R = 4.5 min, k = 4.9). $C_{30}H_{40}N_6O_3 \cdot C_6H_3F_9O_6$ (532.69 + 342.06).

5-(2-(4-Aminopiperidin-1-yl)acetyl)-5,10-dihydro-11*H*-dibenzo[*b*,*e*][1,4]diazepin-11-on bis(hydrotrifluoroacetate) (28)

Compound **8** (314 mg, 1.10 mmol) and compound **26** (592 mg, 2.96 mmol) were suspended in DMF (4.0 mL) followed by the addition of freshly grained potassium carbonate (726 mg, 5.26 mmol). The mixture was stirred at 85 °C under microwave irradiation for 90 min and subsequently cooled to room temperature. Insoluble material was separated by filtration and washed with CH_2Cl_2 (2 × 3 mL). The combined filtrate and washings were concentrated

under reduced pressure yielding a brown residue, which was subjected to preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 57:43, t_R = 15 min) yielding the Boc-protected intermediate **27** as a white solid (522 mg, 99%. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{25}H_{31}N_4O_4]^+$: 451.2340, found: 451.2344). The latter was dissolved in TFA/CH_2Cl_2/H_2O (1:4:0.1) and the mixture was stirred at room temperature for 3 h. CH_2Cl_2 (20 mL) was added and the volatiles were evaporated under reduced pressure. This was repeated once. The residue was dissolved in H_2O and subjected to lyophilization, yielding compound **28** (543 mg, 87%) as a pale yellow solid. R_f = 0.1 (CH_2Cl_2/MeOH 9:1 v/v). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.66-1.92 (m, 2H), 1.92-2.13 (m, 2H), 2.97-3.22 (m, 2H), 3.39 (s, 1H), 3.54-3.95 (m, 2H), 4.36 (s, 2H), 7.20-7.30 (m, 1.4H), 7.31-7.38 (m, 1H), 7.40-7.48 (m, 1H), 7.48-7.56 (m, 1.2H), 7.58 (t, *J* 7.6 Hz, 0.4H), 7.65-7.84 (m, 2.6H), 7.87 (d, *J* 7.6 Hz, 0.4H), 8.19 (s, 3H), 10.04 (brs, 1H), 10.72 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 27.06, 45.15, 50.67, 51.18, 55.82, 116.82 (q, *J* 300 Hz, TFA), 121.84, 122.27, 124.84, 125.42, 126.66, 127.23, 127.65, 128.24, 128.68, 128.92, 129.50, 129.68, 129.97, 130.38, 130.92, 131.50, 133.00, 133.70, 134.68, 135.75, 140.99, 158.20 (q, *J* 32 Hz, carbonyl group of TFA), 165.75, 166.02. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{20}H_{23}N_4O_2]^+$: 351.1816, found: 351.1841. RP-HPLC (220 nm): 88% (t_R = 4.3 min, *k* = 4.7). $C_{20}H_{22}N_4O_2 \cdot C_4H_2F_6O_4$ (350.42 + 228.04).

2-Amino-N-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)acetamide bis(hydrotrifluoroacetate) (31)

The synthesis of **31** was performed in a 2 mL polypropylene reaction vessel with screw cap. HOBt (34 mg, 0.225 mmol) and EDC × HCl (43 mg, 0.225 mmol) were added to a solution of compound **28** (65 mg, 0.112 mmol) and **29** (39 mg, 0.225 mmol) in DMF (0.5 mL). DIPEA (49 µL, 0.281 mmol) was immediately added and the vessel was shaken at 5 °C overnight. 10% aq TFA (216 µL, ca. 0.3 mmol) was added. The mixture was subjected to preparative HPLC (gradient: 0-25 min: 0.1% aq. TFA/acetonitrile 85:15 to 57:43, t_R = 14 min) to isolate the Boc-protected intermediate **30** (42 mg, 60%. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{27}H_{34}N_5O_5]^+$: 508.2554, found: 508.2563). Compound **30** (57 mg, 0.0918 mmol) was dissolved in TFA/H_2O (95:5) and the mixture was stirred at room temperature for 3 h. CH_2Cl_2 (20 mL) was added and the volatiles were evaporated under reduced pressure. This was repeated once. The residue was dissolved in H_2O and subjected to lyophilization, yielding compound **31** as a white solid (47 mg, 48%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (400.3 MHz, MeOH-d_4): δ (ppm) 1.76-1.96 (m, 2H), 2.06-

2.22 (m, 2H), 3.06-3.28 (m, 2H), 3.45-3.59 (m, 1H), 3.66 (s, 2H), 3.71-3.89 (m, 2H), 3.89-4.01 (m, 1H), 4.47 (t, *J* 17 Hz, 1H), 7.22-7.43 (m, 2.4H), 7.45-7.57 (m, 2.2H), 7.61-7.73 (m, 2H), 7.60 (t, *J* 7.8 Hz, 0.4H), 7.91 (d, *J* 7.8 Hz, 0.6H), 7.98 (d, *J* 7.5 Hz, 0.4H). ^{13}C-NMR (100.7 MHz, MeOH-d_4): δ (ppm) 27.06, 28.37, 40.05, 122.25,125.44, 126.12, 127.08, 127.47, 128.05, 128.73, 129.18, 129.48, 130.34, 130.97, 131.65, 131.96, 133.18,139.57, 165.56. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{22}H_{26}N_5O_3]^+$: 408.2030, found: 408.2036. RP-HPLC (220 nm): 99% (t_R = 4.4 min, *k* = 4.8). $C_{22}H_{25}N_5O_3 \cdot C_4H_2F_6O_4$ (407.47 + 228.04).

2-Chloro-N-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)acetamide (hydrotrifluoroacetate) (33)

The synthesis of **33** was performed in a round bottom flask. DIPEA (110 µL, 0.657 mmol) was added to a solution of compound **28** (152 mg, 0.263 mmol) and **32** (89 mg, 0.789 mmol) in DMF (5 mL) and the resulting solution was stirred at room temperature for 2 h. The product was purified by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 66:34, t_R = 11 min) yielding compound **33** as a pale yellow solid (98 mg, 88%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (400.3 MHz, MeOH-d_4): δ (ppm) 1.76-1.96 (m, 2H), 2.05-2.22 (m, 2H), 3.01-3.28 (m, 2H), 3.41-3.58 (m, 1H), 3.71-3.98 (m, 3H), 4.01 (s, 2H), 4.46 (t, *J* 16 Hz, 1H), 7.24-7.42 (m, 2.4H), 7.45-7.57 (m, 2.2H), 7.60-7.73 (m, 2H), 7.73-7.80 (m, 0.4H), 7.91 (d, *J* 8.2 Hz, 0.6H), 7.98 d, *J* 7.7 Hz, 0.4H). ^{13}C-NMR (100.7 MHz, MeOH-d_4): δ (ppm) 28.25, 121.69, 122.26, 125.44, 126.12, 126.45, 127.08, 127.47, 128.05, 128.73, 129.17, 129.48, 129.84, 130.35, 130.60, 130.97, 133.18, 133.50, 134.06, 134.35, 135.68, 141.24, 163.48, 163.93, 167.41, 167.69. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{22}H_{24}ClN_4O_3]^+$: 427.1531, found: 427.1540. $C_{22}H_{23}ClN_4O_3 \cdot C_2HF_3O_2$ (426.90 + 114.02).

2-(4-(2-Aminoethyl)piperazin-1-yl)-N-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)acetamide tetrakis(hydrotrifluoroacetate) (35)

Compound **28** (61.8 mg, 0.145 mmol), **11** (56.4 mg, 0.246 mmol) and potassium carbonate (100 mg, 0.723 mmol) were suspended in acetonitrile (4 mL) and the mixture was stirred at 85 °C under microwave irradiation for 90 min. After cooling to room temperature, insoluble material was separated by filtration and washed with acetonitrile (2 × 10 mL) and CH_2Cl_2 (2 × 5 mL). The combined filtrate and washings were concentrated under reduced pressure yielding the pale brown residue, which was subjected to preparative HPLC (gradient: 0-30

min: 0.1% aq. TFA/acetonitrile 85:15 to 57:43, t_R = 11 min) yielding the Boc-protected intermediate **34** (82 mg, 67%. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{33}H_{46}N_7O_5]^+$: 620.3555, found: 620.3568). **34** (76 mg, 0.122 mmol) was dissolved in TFA/H_2O (95:5). The mixture was stirred at room temperature for 3 h, CH_2Cl_2 (20 mL) was added and the volatiles were evaporated under reduced pressure. This was repeated once. The residue was subjected to preparative HPLC (gradient: 0-30 min: 85:15 to 66:34, t_R = 7 min) to give compound **35** as a white solid (64 mg, 53%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, MeOH-d_4): δ (ppm) 1.79-1.96 (m, 2H), 2.06-2.22 (m, 2H), 2.74 (t, *J* 5.8 Hz, 2.6H), 2.84 (brs, 3.2H), 3.09 (t, *J* 5.6 Hz, 2.6H), 3.15-3.28 (m, 1.6H), 3.39 (brs, 3.6H, interfering with solvent residual peak), 3.46-3.58 (m, 1.4H), 3.71-3.88 (m, 2H), 3.34 (s, 3H), 4.47 (t, *J* 17 Hz, 1H), 7.23-7.41 (m, 2.4H), 7.46-7.55 (m, 2.2H), 7.61-7.72 (m, 2H), 7.73-7.79 (m, 0.4H), 7.90 (d, *J* 8.2 Hz, 0.6H), 7.97 (d, *J* 7.8 Hz, 0.4H). ^{13}C-NMR (100.7 MHz, MeOH-d_4): δ (ppm) 37.19, 50.38, 53.78, 54.62, 57.84, 123.07, 123.66, 126.84, 127.53, 128.48, 128.86, 129.45, 130.12, 130.57, 130.87, 131.23, 131.73, 131.97, 132.35, 133.03, 133.35, 134.58, 135.48, 137.04, 140.99, 142.65, 165.15, 165.32, 168.82. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{28}H_{38}N_7O_3]^+$: 520.3031, found: 520.3032. RP-HPLC (220 nm): 97% (t_R = 4.2 min, *k* = 4.5). $C_{28}H_{37}N_7O_3 \cdot C_8H_4F_{12}O_8$ (519.65 + 456.08).

Benzyl 3-(4-(2-((tert-butoxycarbonyl)amino)ethyl)piperazin-1-yl)propanoate (37)

Compound **11** (173 mg, 0.754 mmol), **36** (165 mg, 0.830 mmol) and freshly grained potassium carbonate (521 mg, 3.77 mmol) were suspended in acetonitrile (5 mL) and the mixture was stirred at 85 °C under microwave irradiation for 90 min, and then cooled to room temperature. Insoluble material was removed by filtration and washed with acetonitrile (2 × 10 mL) and CH_2Cl_2 (2 × 5 mL). The filtrate and washings were combined and concentrated under reduced pressure yielding a brown residue, which was purified by flash chromatography (gradient: 0-20 min: CH_2Cl_2/MeOH 95:5 to 85:15) to afford **37** as a pale brown solid (283 mg, 96%). R_f = 0.4 (CH_2Cl_2/MeOH 9:1 v/v). ^{1}H-NMR (400.3 MHz, MeOH-d_4): δ (ppm) 1.43 (s, 9H), 2.41-2.61 (m, 12H), 2.70 (t, *J* 6.9 Hz, 2H), 3.18 (t, *J* 6.8 Hz, 2H), 5.13 (s, 2H), 7.28-7.39 (m, 5H). ^{13}C-NMR (100.7 MHz, MeOH-d_4): δ (ppm) 27.36, 31.06, 52.15, 52.20, 52.43, 53.41, 57.24, 65.92, 78.67, 126.59, 126.86, 127.81, 127.91, 127.95, 128.13, 136.21, 156.96, 172.28. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{21}H_{34}N_3O_4]^+$: 392.2544, found 392.2556. $C_{21}H_{33}N_3O_4$ (391.51).

3-(4-(2-((Tert-butoxycarbonyl)amino)ethyl)piperazin-1-yl)propanoic acid (38)

Compound **37** (285 mg, 0.773 mmol) was dissolved in MeOH (7 mL) in a two-necked round bottom flask and 10 % Pd/C (60 mg) was added. The mixture was stirred under an atmosphere of hydrogen at room temperature for 90 min. The catalyst was removed by centrifugation and additional filtration of the supernatant through a 0.2 µm nylon syringe filter. The catalyst was washed with MeOH (2 × 5 mL). The combined washings and filtrate were concentrated under reduced pressure and the residue was suspended in H_2O and subjected to lyophilzation to give compound **38** as a pale yellow solid (197 mg, 90%), which was used without further purification. R_f = 0.1 (CH_2Cl_2/MeOH 9:1 v/v). ^{1}H-NMR (400.3 MHz, MeOH-d_4): δ (ppm) 1.44 (s, 9H), 2.47-2.59 (m, 4H), 2.66-2.82 (m, 4H), 3.09-3.25 (m, 8H). ^{13}C-NMR (100.7 MHz, MeOH-d_4): δ (ppm) 27.34, 29.81, 36.94, 50.44, 51.26, 53.56, 56.50, 78.73, 176.23. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{14}H_{28}N_3O_4]^+$: 302.2074, found 302.2084. $C_{14}H_{27}N_3O_4$ (301.39).

3-(4-(2-Aminoethyl)piperazin-1-yl)-N-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propanamide tetrakis(hydrotrifluoroacetate) (40)

The synthesis of **40** was performed in 2 mL polypropylene reaction vessels with screw cap. HOBt (22 mg, 0.083 mmol) and EDC × HCl (16 mg, 0.083mmol) were added to a solution of compound **38** (25 mg, 0.083 mmol) and **28** (40 mg, 0.069 mmol) in DMF (0.4mL). DIPEA (30 µL, 0.173 mmol) was immediately added and the solution was shaken at 55 °C overnight. 10% aq TFA (170 µL, ca. 0.2 mmol) was added and the product was purified by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 57:43, t_R = 13 min) yielding the Boc-protected intermediate **39** as a pale yellow solid (31 mg, 70%). Compound **39** (112 mg, 0.177 mmol. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{34}H_{48}N_7O_5]^+$: 634.3711, found: 634.3720) was dissolved in TFA/H_2O (95:5) and the mixture was stirred at room temperature for 3 h. CH_2Cl_2 (20 mL) was added and the volatiles were evaporated under reduced pressure. This was repeated once. The residue was purified by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 66:34, t_R = 7min) to give compound **40** as a white solid (107 mg, 61%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.62-1.79 (m, 2H), 1.83-1.90 (m, 1H), 1.90-2.01 (m, 1H), 2.30-2.48 (m, 2H), 2.54-2-65 (m, 4H), 2.86-2.99 (m, 5H), 3.01-3.20 (m, 2H), 3.29 (t, *J* 6.7 Hz, 3H), 3.33-3.54 (m, 3H), 3.65 (d, *J* 44 Hz, 2H), 3.78 (d, *J* 16 Hz, 0.6H), 3.92 (d, *J* 16 Hz, 0.4H), 4.30-4.42 (m, 1H), 7.20-7.31 (m, 1.6H), 7.33-

7.38 (m, 1H), 7.42-7.49 (m, 1H), 7.50-7.55 (m, 1.4H), 7.60 (t, *J* 7.7 Hz, 0.4H), 7.66-7.76 (m, 1.8H), 7.77-7.91 (m, 4.8H), 8.34 (d, *J* 7.2 Hz, 1H), 9.92 (brs, 1H, interfering with signal at 10.12 ppm), 10.12 (brs, 1H, interfering with signal at 9.92 ppm), 10.74 (s, 0.4H), 10.80 (s, 0.6H)^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 28.47, 29.43, 35.60, 43.91, 48.90, 51.04, 51.62, 52.14, 53.11, 55.73, 116.86 (q, *J* 299 Hz, TFA), 121.89, 122.34, 124.88, 125.48, 126.74, 127.27, 127.70, 128.27, 128.71, 128.97, 129.58, 129.72, 130.03, 130.41, 130.95, 131.56, 131.64, 133.04, 133.40, 134.71, 135.80, 139.48, 140.01, 158.40 (q, *J* 32 Hz, carbonyl group of TFA), 164.16, 165.75, 166.04, 168.30. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{29}H_{40}N_7O_3]^+$: 534.3187, found 534.3197. RP-HPLC (220 nm): 99% (t_R = 4.0 min, *k* = 4.3). $C_{29}H_{39}N_7O_3 \cdot C_8H_4F_{12}O_8$ (533.68 + 456.08).

5-(2-(4-(4-(4-aminopiperidin-1-yl)butyl)piperidin-1-yl)acetyl)-5,10-dihydro-11*H*-dibenzo[*b*,*e*][1,4]diazepin-11-one tris(hydrotrifluoroacetate) (43)

Compound **41** (202 mg, 0.430 mmol) and compound **26** (232 mg, 1.16 mmol) were suspended in acetonitrile (4 mL) and potassium carbonate (297 mg, 2.15 mmol) was added. The mixture was stirred at 85 °C under microwave irradiation for 90 min and cooled to room temperature. Insoluble material was separated by filtration and washed with acetonitrile (2 × 4 mL). The combined filtrate and washings were concentrated under reduced pressure yielding a pale yellow residue. Purification by flash chromatography (gradient: 0-20 min: CH_2Cl_2/MeOH 95:5 to 85:15) gave the Boc-protected intermediate **42** as a white solid (235 mg, 99%. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{34}H_{48}N_5O_4]^+$: 590.3701, found: 590.3711). R_f = 0.3 (CH_2Cl_2/MeOH 9:1 v/v). Compound **42** (235 mg, 0.398 mmol) was dissolved in CH_2Cl_2/TFA/H_2O 4:1:0.1 (v/v/v 3 mL). The solution was stirred at room temperature for 3 h followed by evaporation of the volatiles. CH_2Cl_2 (20 mL) was added and the volatiles were evaporated under reduced pressure. This was repeated once. The product was purified by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 66:34, t_R = 7 min) yielding compound **43** as a white solid (294 mg, 89%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.11-1.50 (m, 7H), 1.54-1.64 (m, 2H), 1.66-1.91 (m, 4H), 1.93-2.15 (m, 2H), 2.84-3.09 (m, 6H), 3.22-3.31 (m, 2H), 3.49 (d, *J* 12 Hz, 2H), 3.58 (d, *J* 11 Hz, 1H), 3.67 (d, *J* 17 Hz, 0.6H), 3.77 (d, *J* 17 Hz, 0.4H), 4.27-4.38 (m, 1H), 7.20-7.30 (m, 1.4H), 7.32-7.38 (m, 1H), 7.41-7.48 (m, 1H), 7.48-7.54 (m, 1.2H), 7.59 (t, *J* 7.7 Hz, 0.4H), 7.67-7.75 (1.6H), 7.76-7.80 (m, 0.4H), 7.82 d, *J* 7.6 Hz, 0.6H), 7.88 (d, *J* 7.8 Hz, 0.4H), 8.33 (s, 3H), 9.70 (brs, 1H), 10.15 (brs, 1H), 10.74 (s, 0.4H), 10.80 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 23.05, 23.37, 27.20, 28.67, 32.69, 34.69, 45.13, 49.80, 52.73, 53.17, 55.69, 55.94, 117.10 (q, *J*

299 Hz, TFA), 121.89, 122.32, 124.88, 125.48, 126.78, 127.30, 127.73, 128.30,128.69, 128.94, 129.55, 129.75, 129.94, 130.41, 130.94, 131.52, 131.71, 133.03, 133.16, 133.76, 134.72, 135.80, 141.06, 158.47 (q, *J* 31 Hz, carbonyl group of TFA), 165.77, 166.05. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{29}H_{40}N_5O_2]^+$: 490.3177, found 490.3184. RP-HPLC (220 nm): 99% (t_R = 4.5 min, *k* = 4.9). $C_{29}H_{39}N_5O_2 \cdot C_6H_3F_9O_6$ (489.66 + 342.06).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-((2-(((S)-5-guanidino-1-oxo-1-((4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)amino)pentan-2-yl)amino)-2-oxoethyl)amino)-1-oxopropan-2-yl)pentanamide tris(hydrotrifluoroacetate) (53)

Compound **53** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 22 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to -66:34, t_R = 11 min) yielded **53** as a white fluffy solid (11 mg, 0.0085 mmol, 41%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.13-1-26 (m, 7H), 1.32-1.54 (m, 11H), 1.62-1.70 (m, 2H), 1.70-1.83 (m, 2H), 1.85 (s, 3H), 2.85-2.97 (m, 1H), 2.97-3.13 (m, 7H), 3.26 (d, *J* 11 Hz, 1H), 3.58 (d, *J* 11 Hz, 1H), 3.63-3.72 (m, 2.6H), 3.77 (d, *J* 17 Hz, 0.4H), 4.09-4.20 (m, 3H), 4.29 (d, *J* 17 Hz, 0.6H), 4.34 (d, *J* 17 Hz, 0.4H), 7.05 (brs, 4.4H), 7.22-7.31 (m, 2.6H), 7.32-7.39 (m, 2H), 7.39-7.49 (m, 2H), 7.49-7.51 (m, 1.4H), 7.59 (t, *J* 7.5 Hz, 0.6H), 7.65-7.79 (m, 4H), 7.82 (d, *J* 8.1 Hz, 0.6H), 7.88 (d, *J* 7.9 Hz, 0.4H), 7.90-7.94 (m, 2H), 8.04-8.10 (m, 2H), 8.14 (t, *J* 5.7 Hz, 1H), 9.57 (brs, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 18.04, 22.49, 23.26, 24.99, 25.07, 28.73, 29.04, 29.11, 29.30, 32.77, 34.94, 38.45, 41.99, 48.29, 52.12, 52.67, 52.80, 53.25, 55.94, 115.97 (TFA), 117.94 (TFA), 121.90, 122.32, 124.90, 125.48, 126.77, 127.27, 127.71, 128.27, 128.72, 129.58, 129.71, 130.02, 130.38, 130.95, 131.56, 131.63, 133.06, 134.69, 135.78, 139.46, 141.00, 156.80 (2 × guanidinium group), 158.49 (q, *J* 32 Hz, carbonyl group of TFA), 165.75, 166.05, 168.55, 169.58, 170.89, 171.34, 172.55. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{43}H_{65}N_{14}O_7]^+$: 889.5155, found 889.5155. RP-HPLC (220 nm): 99% (t_R = 5.7 min, *k* = 6.5). $C_{43}H_{64}N_{14}O_7 \cdot C_6H_3F_9O_6$ (889.08 + 342.06).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamid tris(hydrotrifluoroacetate) (54)

Compound **54** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 23 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to -66:34, t_R = 11 min) yielded **54** as a white fluffy solid (17 mg, 0.014 mmol, 40%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.23-1.25 (m, 7H), 1.30-1.55 (m, 11H), 1.61-1.69 (m, 2H), 1.70-1.82 (m, 2H), 1.86 (s, 3H), 2.86-2.96 (m, 1H), 2.96-3.14 (m, 7H), 3.26 (d, *J* 11 Hz, 1H), 3.57 (d, *J* 11 Hz, 1H), 3.67 (d, *J* 17 Hz, 0.6H), 3.77 (d, *J* 17 Hz, 0.4H), 4.11-4.26 (m, 3H), 4.34-4.48 (m, 1H), 7.07 (brs, 4H), 7.22-7.31 (m, 2.8H), 7.32-7.41 (m, 2.6H), 7.41-7.48 (m, 1.6H), 7.49-7.55 (m, 1.6H), 7.59 (t, *J* 7.5 Hz, 0.4H), 7.68-7.90 (m, 7H), 8.05 (d, *J* 7.1 Hz, 1H), 8.09 (d, *J* 7.7 Hz, 1H), 9.58 (s, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.88, 22.49, 23.21, 25.01, 25.06, 28.74, 28.90, 29.10, 29.20, 32.77, 34.94, 38.39, 40.34, 40.43, 48.37, 52.25, 52.36, 52.68, 52.80, 53.24, 55.96, 116.16 (TFA), 118.15 (TFA), 121.90, 125.48, 126.76, 127.28, 127.72, 128.26, 128.72, 129.72, 130.01, 130.38, 130.95, 131.54, 133.05, 133.76, 134.70, 135.78, 156.82 (guanidinium group), 158.52 (q, *J* 31 Hz, carbonyl group of TFA), 165.77, 166.06, 169.76, 170.93, 171.44, 171.98. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{41}H_{62}N_{13}O_6]^+$: 832.4941, found 832.4943. RP-HPLC (220 nm): 99% (t_R = 5.7 min, *k* = 6.5). $C_{41}H_{61}N_{13}O_6 \cdot C_6H_3F_9O_6$ (832.02 + 342.06).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-oxo-1-((2-oxo-2-((4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)amino)ethyl)amino)propan-2-yl)pentanamide bis(hydrotrifluoroacetate) (55)

Compound **55** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 17 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to -66:34, t_R = 11 min) yielded **55** as a white fluffy solid (22 mg, 0.023 mmol, 57%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.11-1.26 (m, 7H), 1.30-1.55 (m, 8H), 1.62-1.69 (m, 1H), 1.71-1.77 (m, 1H), 1.77-1.83 (m, 1H), 1.85 (s, 3H), 2.86-2.97 (m, 1H), 2.99-3.13 (m, 5H), 3.33 (d, *J* 11 Hz, 1H), 3.58-3.68 (m, 3H), 3.77 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.19-4.27 (m, 2H), 4.34-4.47 (m, 1H),

7.02 (brs, 2H), 7.21-7.30 (m, 2H), 7.31-7.39 (m, 2H), 7.42-7.49 (m, 1.2H), 7.49-7.54 (m, 1.4H), 7.60 (t, *J* 7.4 Hz, 0.4H), 7.63-7.80 (m, 4H), 7.82 (d, *J* 8.0 Hz, 0.6H), 7.88 (d, *J* 7.9 Hz, 0.4H), 8.03-8.16 (m, 3H), 9.56 (s, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.79, 22.49, 23.22, 24.94, 28.71, 28.99, 29.12, 32.75, 34.93, 38.40, 40.43, 42.05, 48.48, 52.16, 52.66, 52.80, 53.26, 55.95, 115.89 (TFA), 117.87 (TFA), 121.89, 122.32, 124.90, 125.49, 126.77, 127.27, 127.73, 128.27, 128.72, 128.97, 129.58, 129.71, 130.02, 130.38, 130.95, 131.56, 131.63, 133.05, 133.78, 134.69, 135.78, 139.45, 140.99, 156.79 (guanidinium group), 158.20 (carbonyl group of TFA), 158.42 (carbonyl group of TFA), 164.19, 165.75, 166.05, 168.37, 169.58, 171.50, 172.35. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{37}H_{53}N_{10}O_6]^+$: 733.4144, found 733.4151. RP-HPLC (220 nm): 99% (t_R = 6.2 min, *k* = 7.2). $C_{37}H_{52}N_{10}O_6 \cdot C_4H_2F_6O_4$ (732.89 + 228.04).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-(((*R*)-5-guanidino-1-oxo-1-((4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide tris((hydrotrifluoroacetate) (56)

Compound **56** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 23 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to -66:34, t_R = 11 min) yielded **56** as a white fluffy solid (10 mg, 0.0087 mmol, 49%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.13-1.25 (m, 7H), 1.31-1.54 (m, 11H), 1.58-1.82 (m, 4H), 1.86 (s, 3H), 2.86-2.96 (m, 1H), 2.96-3.13 (m, 7H), 3.26 (d, *J* 12 Hz, 1H), 3.57 (d, *J* 12 Hz, 1H), 3.66 (d, *J* 17 Hz, 0.6H), 3.76 (d, *J* 17 Hz, 0.4H), 4.11-4.16 (m, 1H), 4.17-4.27 (m, 2H), 4.34-4.67 (m, 1H), 7.02 (brs, 4H), 7.22-7.31 (m, 3.2H), 7.32-7.41 (m, 2.4H), 7.41-7.49 (m, 1.6H), 7.49-7.55 (m, 1.4H), 7.59 (t, *J* 7.4 Hz, 0.4H), 7.64-7.78 (m, 4H), 7.78-7.84 (m, 1.6H), 7.88 (dd, *J* 7.8, 1.1 Hz, 0.4H), 8.01 (d, *J* 8.2 Hz, 1H), 8.07-8.14 (m, 2H), 9.55 (s, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.92, 22.48, 23.23, 24.96, 25.15, 28.72, 28.85, 29.11, 32.77, 34.94, 38.46, 40.33, 40.40, 48.46, 52.20, 52.32, 52.80, 53.26, 55.95, 115.93 (TFA), 117.90 (TFA), 121.89, 122.32, 124.90, 125.48, 127.26, 127.71, 128.25, 128.73, 129.71, 130.38, 130.95, 131.56, 133.05, 134.69, 135.77, 140.98, 156.78 (2 × guanidinium group), 158.36 (carbonyl group of TFA), 158.58 (carbonyl group of TFA), 165.75, 166.05, 169.77, 170.88, 171.51, 172.11. HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{41}H_{63}N_{13}O_6]^{2+}$: 416.7507, found 416.7516. RP-HPLC (220 nm): 99% (t_R = 5.6 min, *k* = 6.4). $C_{41}H_{61}N_{13}O_6 \cdot C_6H_3F_9O_6$ (832.02 + 342.06).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-oxo-1-((4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)amino)propan-2-yl)pentanamide bis(hydrotrifluoroacetate) (57)

Compound **57** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 17 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to -66:34, t_R = 12 min) yielded **57** as a white fluffy solid (18 mg, 0.019 mmol, 56%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.14-1.26 (m, 7H), 1.31-1.53 (m, 8H), 1.58-1.69 (m, 1H), 1.69-1.77 (m, 1H), 1.77-1.82 (m, 1H), 1.85 (s, 3H), 2.87-3.14 (m, 6H), 3.33 (d, *J* 11 Hz, 1H), 3.54-3.64 (m, 1H), 3.77 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.15-4.25 (m, 2H), 4.34-4.46 (m, 1H), 7.05 (brs, 2H), 7.22-7.41 (m, 4H), 7.41-7.56 (m, 2.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.67-7.80 (m, 4H), 7.82 (d, *J* 8.1 Hz, 0.6H), 7.88 (d, *J* 8.1 Hz, 0.4H), 7.94 (d, *J* 7.5 Hz, 1H), 8.06 (d, *J* 7.7 Hz, 1H), 9.58 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 18.37, 22.52, 23.13, 24.98, 28.71, 29.01, 32.74, 34.91, 38.35, 40.42, 48.17, 52.28, 52.66, 52.80, 53.25, 55.95, 115.84 (TFA), 117.81 (TFA), 121.90, 122.32, 124.90, 125.49, 126.78, 127.27, 127.73, 128.27,128.72, 128.97, 129.58, 129.72, 130.02, 130.28, 130.95, 131.55, 131.64, 133.05, 133.78, 134.70, 135.78, 139.46, 141.00, 156.82 (guanidinium group), 158.38 (q, *J* 33 Hz, carbonyl group of TFA), 163.70, 164.20, 165.75, 166.05, 169.62, 171.05, 171.81. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{35}H_{50}N_9O_5]^+$: 676.3929, found 676.3939. RP-HPLC (220 nm): 99% (t_R = 6.4 min, *k* = 7.4). $C_{35}H_{49}N_9O_5 \cdot C_4H_2F_6O_4$ (675.84 + 228.04).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-5-guanidino-1-oxo-1-((4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)amino)pentan-2-yl)pentanamide tris(hydrotrifluoroacetate) (58)

Compound **58** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 20 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to -66:34, t_R = 10 min) yielded **58** as a white fluffy solid (22 mg, 0.021 mmol, 44%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.13-1.26 (m, 4H), 1.31-1.58 (m, 11H), 1.61-1.70 (m, 2H), 1.71-1.83 (m, 2H), 1.86 (s, 3H), 2.70-2.82 (m, 2H), 2.87-3.13 (m, 6H), 3.29-3.37 (m, 1H), 3.56-3.63 (m, 1H), 3.76 (d, *J* 17 Hz, 0.6H), 3.90 (d, *J* 16 Hz, 0.4H), 4.16-4.21 (m, 1H), 4.22-4.28 (m, 1H), 4.36-4.47 (m, 1H), 7.08 (brs, 2H), 7.22-7.31 (2H), 7.31-7.40 (m, 2H), 7.42-7.49 (m, 1.2H), 7.49-7.55 (m, 1.4H),

7.60 (t, *J* 7.6 Hz, 0.4H), 7.68-7.83 (m, 6.6H), 7.86-7.91 (m, 1.4H), 7.97 (d, *J* 8.0 Hz, 1H), 8.09 (d, *J* 7.9 Hz, 1H), 9.58 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 22.49, 23.26, 23.64, 25.10, 28.72, 29.10, 29.40, 32.77, 34.95, 38.45, 40.33, 51.91, 52.10, 52.80, 53.25, 55.95, 116.88 (q, *J* 300 Hz, TFA), 121.90, 122.32, 124.90, 125.49, 126.76, 127.27, 127.72, 128.26, 128.73, 128.98, 129.59, 129.71, 130.03, 130.38, 130.96, 131.56, 131.63, 133.05, 133.78, 134.69, 135.78, 141.00, 156.82 (guanidinium group), 158.38 (q, *J* 32 Hz, carbonyl group of TFA), 164.19, 165.76, 166.05, 169.65, 170.89, 171.41. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{37}H_{55}N_{10}O_5]^+$: 719.4351, found 719.4357. RP-HPLC (220 nm): 97% (t_R = 5.0 min, *k* = 5.6). $C_{37}H_{54}N_{10}O_5 \cdot C_6H_3F_9O_6$ (718.90 + 342.06).

(*R*)-2-Acetamido-5-amino-N-((*S*)-5-guanidino-1-oxo-1-((4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)amino)pentan-2-yl)pentanamide tris(hydrotrifluoroacetate) (59)

Compound **59** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 20 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to -66:34, t_R = 10 min) yielded **59** as a white fluffy solid (20 mg, 0.019 mmol, 40%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.12-1.26 (m, 4H), 1.29-1.59 (m, 11H), 1.59-1.66 (m, 1H), 1.66-1.76 (m, 2H), 1.76-1.83 (m, 1H), 1.88 (s, 3H), 2.72-2.85 (m, 2H), 2.85-2.96 (m, 1H), 2.96-3.12 (m, 5H), 3.29-3.36 (m, 1H), 3.55-3.62 (m, 1H), 3.76 (d, *J* 17 Hz, 0.6H), 3.90 (d, *J* 17 Hz, 0.4H), 4.14-4.18 (m, 1H), 4.22-4.26 (m, 1H), 4.36-4.46 (m, 1H), 7.13 (brs, 2H), 7.22-7.32 (m, 2H), 7.33-7.39 (m, 1.6H), 7.39-7.49 (m, 1.4H), 7.49-7.56 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.67-7.90 (m, 8H), 8.15 (d, *J* 7.7 Hz, 1H), 8.22 (d, *J* 8.3 Hz, 1H), 9.58 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 22.42, 23.23, 23.59, 25.15, 28.69, 29.04, 29.12, 32.76, 34.96, 38.40, 38.49, 40.34, 52.15, 52.68, 52.81, 53.26, 55.95, 116.79 (q, *J* 298 Hz, TFA), 121.90, 122.33, 124.91, 125.49, 126.78, 127.28, 127.73, 128.27, 128.73, 128.98, 129.58, 129.72, 130.02, 130.39, 130.96, 131.56, 131.64, 133.06, 133.11, 133.78, 134.70, 135.78, 139.46, 141.01, 156.87 (guanidinium group), 158.45 (q, *J* 32 Hz, carbonyl group of TFA), 163.70, 164.19, 165.76, 166.06, 169.76, 170.86, 171.59. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{37}H_{55}N_{10}O_5]^+$: 719.4351, found 719.4361. RP-HPLC (220 nm): 98% (t_R = 5.2 min, *k* = 9.0). $C_{37}H_{54}N_{10}O_5 \cdot C_6H_3F_9O_6$ (718.90 + 342.06).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-4-guanidino-1-oxo-1-((4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)amino)butan-2-yl)pentanamidetris(hydrotrifluoroacetate) (60)

Compound **60** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 20 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to -66:34, t_R = 9 min) yielded **60** as a white fluffy solid (15 mg, 0.014 mmol, 48%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.12-1.26 (m, 4H), 1.29-1.46 (m, 5H), 1.50-1.63 (m, 3H), 1.65-1.76 (m, 3H), 1.76-1.83 (m, 1H), 1.83-1.93 (m, 4H), 2.72-2.83 (m, 2H), 2.85-2.96 (m, 1H), 2.96-3.15 (m, 5H), 3.29-3.36 (m, 1H), 3.58 (d, *J* 11 Hz, 1H), 3.76 (d, *J* 17 Hz, 0.6H), 3.90 (d, *J* 17 Hz, 0.4H), 4.15-4.26 (m, 2H), 4.35-4.48 (m, 1H), 7.32 (brs, 4H, interfering with following signal), 7.33-7.41 (m, 2H), 7.41-7.49 (m, 1H), 7.49-7.57 (m, 1.6H), 7.60 (t, *J* 7.6 Hz, 0.4H), 7.67-7.92 (m, 8H), 8.12 (d, *J* 8.1 Hz, 1H), 8.17 (d, *J* 7.5 Hz, 1H), 9.60 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 22.50, 23.23, 23.66, 28.48, 28.73, 29.05, 31.53, 32.75, 34.96, 37.89, 38.48, 50.30, 52.29, 52.66, 52.80, 53.24, 55.95, 116.98 (q, *J* 299 Hz, TFA), 121.90, 122.33, 124.91, 125.49, 126.77, 127.27, 128.27, 128.73, 129.72, 130.02, 130.39, 130.95, 131.55, 131.64, 133.06, 133.11, 133.78, 134.70, 135.78, 139.45, 141.00, 156.92 (guanidinium group), 158.47 (q, *J* 32 Hz, carbonyl group of TFA) 163.68, 164.20, 165.76, 166.06, 169.98, 170.65, 171.69. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{36}H_{53}N_{10}O_5]^+$: 705.4195, found 705.4197. RP-HPLC (220 nm): 97% (t_R = 4.9 min, *k* = 5.4). $C_{36}H_{53}N_{10}O_5 \cdot C_6H_3F_9O_6$ (704.88 + 342.06).

(*S*)-2-Acetamido-6-amino-*N*-((*S*)-5-guanidino-1-oxo-1-((3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b,e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl)amino)pentan-2-yl)hexanamide tris(hydrotrifluoroacetate) (63)

Compound **63** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 18 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to -66:34, t_R = 8 min) yielded **63** as a white fluffy solid (14 mg, 0.013 mmol, 47%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.16 (s, 2H), 1.24-1.54 (m, 13H), 1.57-1.69 (m, 2H), 1.69-1.81 (m, 2H), 1.85 (s, 3H), 2.71-2.79 (m, 2H), 2.89-3.11 (m, 6H), 3.22-3.30 (m, 1H, interfering with H_2O peak), 3.59 (s, 1H), 3.76 (d, *J* 15 Hz, 0.6H), 3.90 (d, *J* 14 Hz, 0.4H), 4.11-4.22 (m, 2H), 4.33-4.50 (m, 1H), 7.07

(brs, 2H); 7.22-7.40 (m, 4H), 7.40-7.54 (m, 3H), 7.59 (t, *J* 7.4 Hz, 0.4H), 7.68-7.83 (m, 6.6H), 7.85-7.89 (m, 1H), 7.92 (d, J 8.0 Hz, 1H), 8.05 (d, *J* 7.7 Hz, 1H), 9.59 (s, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 22.41, 22.51, 25.13, 25.89, 56.62, 28.70, 29.23, 31.11, 32.45, 38.53, 38.71, 40.32, 52.09, 52.65, 53.20, 55.91, 63.94, 116.23 (TFA), 118.23 (TFA), 121.89, 122.30, 124.90, 125.48, 126.73, 127.28, 127.71, 128.72, 129.72, 130.39, 130.95, 131.59, 133.05, 134.70, 135.77, 141.00, 156.81 (guanidinium group), 158.34 (q, *J* 31 Hz, carbonyl group of TFA), 166.07, 169.69, 171.02, 171.72. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{37}H_{55}N_{10}O_5]^+$: 719.4351, found 719.4366. RP-HPLC (220 nm): 99% (t_R = 4.7 min, *k* = 5.2). $C_{37}H_{54}N_{10}O_5 \cdot C_6H_3F_9O_6$ (718.90 + 342.06).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-5-guanidino-1-oxo-1-((3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl)amino)pentan-2-yl)pentanamide tris(hydrotrifluoroacetate) (64)

Compound **64** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 20 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to -66:34, t_R = 10 min) yielded **64** as a white fluffy solid (21 mg, 0.020 mmol, 36%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.11-1.21 (m, 2H), 1.33-1.60 (m, 11H), 1.61-1.69 (m, 2H), 1.69-1.83 (m, 2H), 1.86 (s, 3H), 2-72-2.82 (m, 2H), 2.87-3.13 (m, 6H), 3.30-3.37 (m, 1H), 3.55-3-63 (m, 1H), 3.76 (d, *J* 16 Hz, 0.6H), 3.91 (d, *J* 16 Hz, 0.4H), 4.15-4.21 (m, 1H), 4.21-4.28 (m, 1H), 4.36-4.48 (m, 1H), 7.09 (brs, 2H), 7.22-7.31 (m, 2H), 7.32-7.41 (m, 1.6H), 7.41-7.49 (m, 1.4H), 7.49-7.57 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.68-7.84 (m, 6.6H), 7.86-7.92 (m, 1.4H), 7.98 (d, *J* 8.1 Hz, 1H), 8.10 (d, *J* 7.9 Hz, 1H), 9.60 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 22.49, 23.65, 25.11, 25.90, 28.71, 29.36, 32.44, 38.44, 38.53, 40.32, 51.92, 52.12, 52.61, 52.75, 53.21, 55.94, 116.75 (q, *J* 297 Hz, TFA), 124.90, 125.48, 126.76, 127.26, 127.71, 128.26, 128.72, 128.98, 129.59, 129.71, 130.02, 130.38, 130.95, 131.56, 131.63, 133.05, 133.78, 134.69, 135.78, 139.45, 140.99, 156.82 (guanidinium group), 158.40 (q, *J* 32 Hz, carbonyl group of TFA), 163.69, 164.18, 165.75, 166.05, 169.66, 170.93, 171.43. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{36}H_{53}N_{10}O_5]^+$: 705.4195, found 705.4206. RP-HPLC (220 nm): 99% (t_R = 4.4 min, *k* = 4.8). $C_{36}H_{52}N_{10}O_5 \cdot C_6H_3F_9O_6$ (704.88 + 342.06).

(*R*)-2-Acetamido-5-amino-*N*-((*S*)-5-((diaminomethylene)amino)-1-oxo-1-((3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl)amino)pentan-2-yl)pentanamide tris(hydrotrifluoroacetate) (65)

Compound **65** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 20 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to -66:34, t_R = 10 min) yielded **65** as a white fluffy solid (22 mg, 0.021 mmol, 46%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.10-1.20 (m, 2H), 1.31-1.60 (m, 11H), 1.60-1.66 (m, 1H), 1.67-1.82 (m, 3H), 1.85 (s, 3H), 2.72-2.83 (m, 2H), 2.87-2.95 (m, 1H), 2.96-3.12 (m, 5H), 3.28-3.37 (m, 1H), 3.55-3.64 (m, 1H), 3.76 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.13-4.18 (m, 1H), 4.22-4.26 (m, 1H), 4.36-4.47 (m, 1H), 7.12 (brs, 2H), 7.22-7.32 (m, 2H), 7.32-7.40 (m, 1.6H), 7.41-7.48 (m, 1.4H), 7.48-7.55 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.67-7.86 (m, 6.6H), 7.86-7.91 (m, 1.4H), 8.15 (d, *J* 7.5 Hz, 1H), 8.23 (d, *J* 8.2 Hz, 1H), 9.60 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 22.41, 23.59, 25.15, 25.84, 28.67, 29.06, 32.40, 38.39, 38.57, 40.33, 52.16, 52.62, 52.76, 53.21, 55.95, 116.99 (q, *J* 298 Hz, TFA), 121.89, 122.32, 124.89, 125.48, 126.76, 127.26, 127.72, 128.26, 128.72, 128.97, 129.56, 129.71, 130.01, 130.38, 130.95, 131.54, 131.63, 133.04, 133.10, 133.78, 134.69, 135.78, 139.45, 141.00, 156.86 (guanidinium group), 158.42 (q, *J* 32 Hz, carbonyl group of TFA), 164.18, 165.75, 166.04, 169.76, 170.91, 171.59. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{36}H_{53}N_{10}O_5]^+$: 705.4195, found 705.4206. RP-HPLC (220 nm): 99% (t_R = 4.5 min, *k* = 4.9). $C_{36}H_{52}N_{10}O_5 \cdot C_6H_3F_9O_6$ (704.88 + 342.06).

(*R*)-2-Amino-5-guanidino-N-(2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)pentanamide pentakis(hydrotrifluoroacetate) (73)

Compound **73** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 17 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 90:10-76:24, t_R = 17 min) yielded **73** as a white fluffy solid (10 mg, 0.0079 mmol, 46%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.16-1.28 (m, 4H), 1.32-1.45 (m, 3H), 1.45-1.52 (m, 2H), 1.54-1.61 (m, 2H), 1.65-1.71 (m, 2H), 1.71-1.75 (m, 1H), 1.75-1.82 (m, 1H), 2.52-2.61 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.77-3.04 (m, 7H), 3.04-3.18 (m, 4H), 3.18-3.53 (m, 6H), 3.54-

3.62 (m, 1H), 3.70-3.80 (m, 1.6H), 3.90 (d, *J* 16 Hz, 0.4H), 4.34-4.46 (m, 1H), 7.08-7.30 (m, 4H), 7.30-7.39 (m, 2H), 7.40-7.48 (m, 2H), 7.48-7.55 (m, 1.6H), 7.59 (t, *J* 7.5 Hz, 0.4H), 7.67-7.77 (m, 2H), 7.81 (d, *J* 8.2 Hz, 0.6H), 7.88 (d, *J* 7.8 Hz, 0.4H), 7.91-7.97 (m, 1H), 8.20 (s, 3H), 8.55 (s. 1H), 9.58 (s, 1H), 10.06 (brs, 1H), 10.72 (s, 0.4H), 10.77 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 23.05, 23.47, 24.13, 28.24, 28.62, 32.74, 34.75, 49.20, 50.54, 51.91, 52.58, 52.73, 55.34, 55.93, 116.56 (q, *J* 297 Hz, TFA), 121.89, 122.31, 124.89, 125.47, 127.26, 127.71, 128.26, 128.72, 128.98, 129.70, 130.02, 130.37, 130.95, 131.65, 131.61, 133.04, 133.77, 134.69, 135.78, 139.44, 140.98, 156.89 (guanidinium group), 158.45 (q, *J* 33 Hz, carbonyl group of TFA), 164.18, 165.74, 166.04, 168.44. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{36}H_{55}N_{10}O_3]^+$: 675.4453, found 675.4457. RP-HPLC (220 nm): 99% (t_R = 4.3 min, *k* = 4.7). $C_{36}H_{54}N_{10}O_3 \cdot C_{10}H_5F_{15}O_5$ (674.90 + 570.10).

(*S*)-2-Amino-5-guanidino-*N*-(2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)pentanamide pentakis(hydrotrifluoroacetate) (74)

Compound **74** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 17 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 10 min) yielded **74** as a white fluffy solid (10 mg, 0.0083 mmol, 39%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.15-1.30 (m, 4H), 1.30-1.44 (m, 3H), 1.46-1.52 (m, 2H), 1.55-1.62 (m, 2H), 1.65-1.72 (m, 2H), 1.72-1.77 (m, 1H), 1.77-1.83 (m, 1H), 2.5-2.65 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.86-2.96 (m, 2H), 2.96-3.07 (m, 4H), 3.11 (q, *J* 6.4 Hz, 4H), 3.22-3.31 (m, 1.6H), 3.31-3.39 (m, 2.4H), 3.40-3.53 (m, 1.6H), 3.56-3.64 (m, 1.4H), 3.69-3.82 (m, 2.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.36-4.47 (m, 1H), 7.11-7.31 (m, 4H), 7.31-7.40 (m, 2H), 7.40-7.49 (m, 2H), 7.49-7.57 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.67-7.79 (m, 2H), 7.82 (d, *J* 8.1 Hz, 0.6H), 7.88 (d, *J* 7.9 Hz, 0.4H), 7.96 (s, 1H), 8.23 (s, 3H), 8.59 (s, 1H), 9.61 (s, 1H), 10.73 (s, 0.4H), 10.76 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 23.04, 23.45, 24.14, 28.24, 28.64, 32.74, 34.75, 35.51, 49.15, 50.35, 51.93, 52.60, 52.74, 53.19, 55.28, 55.93, 116.61 (q, *J* 296 Hz, TFA), 121.90, 122.33, 125.49, 126.78, 127.27,127.73, 128.27, 128.73, 128.99, 129.59, 129.72, 130.03, 130.39, 130.96, 131.63, 133.05, 133.78, 134.70, 135.79, 139.46, 141.00, 156.93 (guanidinium group), 158.48 (carbonyl group of TFA),158.69 (carbonyl group of TFA), 163.70, 164.19, 165.76, 166.05, 168.49. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{36}H_{55}N_{10}O_3]^+$:

675.4453, found 675.4453. RP-HPLC (220 nm): 99% (t_R = 4.5 min, k = 4.9). $C_{36}H_{54}N_{10}O_3 \cdot C_{10}H_5F_{15}O_5$ (674.90 + 570.10).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (75)

Compound **75** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 19 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 11 min) yielded **75** as a white fluffy solid (5.9 mg, 0.0039 mmol, 18%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.12-1.28 (m, 7H), 1.29-1.60 (m, 11H), 1.60-1.69 (m, 2H), 1.69-1.76 (m, 1H), 1.76-1.81 (m, 1H), 1.85 (s, 3H), 2.55-2.69 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.82-3.03 (m, 6H), 3.03-3.14 (m, 6H), 3.14-3.36 (m, 5H), 3.36-3.50 (m, 2H), 3.57-3.61 (m, 1H), 3.76 (d, *J* 16 Hz, 0.6H), 3.90 (d, *J* 16 Hz, 0.4H), 4.11-4.16 (m, 1H), 4.16-4.20 (m, 1H), 4.22-4.26 (m, 1H), 4.35-4.45 (1H), 7.08 (brs, 4H), 7.21-7.31 (m, 3H), 7.31-7.39 (m, 2.4H), 7.39-7.48 (m, 2.2H), 7.48-7.56 (m, 1.6H), 7.59 (t, *J* 7.6 Hz, 0.4H), 7.66-7.85 (m, 5H), 7.85-7.98 (m, 2.4H), 8.02-8.06 (m, 1H), 8.09 (d, *J* 7.5 Hz, 1H), 9.59 (s, 1H), 10.72 (s, 0.4H), 10.77 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.84, 22.49, 23.06, 23.53, 25.01, 28.64, 28.87, 28.97, 32.74, 34.76, 40.37, 40.43, 48.33, 49.23, 50.17, 52.35, 52.41, 52.74, 53.19, 55.34, 55.94, 115.74 (TFA), 117.70 (TFA), 121.91, 122.33, 124.91, 125.49, 126.76, 127.27, 127.73, 128.27, 128.74, 129.03, 129.58, 129.72, 130.04, 130.39, 130.96, 131.63, 133.06, 133.79, 134.70, 135.78, 139.45, 140.99, 156.85 (2 × guanidinium group), 158.51 (carbonyl group of TFA), 158.86 (carbonyl group of TFA), 164.19, 169.82, 171.41, 171.46, 172.15. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{47}H_{74}N_{15}O_6]^+$: 944.5941, found 944.5933. RP-HPLC (220 nm): 99% (t_R = 4.7 min, k = 5.2). $C_{47}H_{73}N_{15}O_6 \cdot C_{10}H_5F_{15}O_5$ (944.20 + 570.10).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-((2-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-2-oxoethyl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (76)

Compound **76** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 19 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 11 min) yielded **76** as a white fluffy solid (5.4 mg, 0.0034 mmol, 15%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.16-1.29 (m, 7H), 1.31-1.53 (m, 9H), 1.53-1.60 (m, 2H), 1.61-1.71 (m, 2H), 1.71-1.77 (m, 1H), 1.77-1.83 (m, 1H), 1.86 (s, 3H), 2.57-2.64 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.85-3.04 (m, 6H), 3.04-3.15 (m, 6H), 3.19-3.40 (m, 7H), 3.56-3.62 (m, 1H), 3.70-3.79 (m, 2.6H), 3.92 (d, *J* 17 Hz, 0.4H), 4.18-4.27 (m, 3H), 4.37-4.46 (m, 1H), 7.05 (brs, 4H), 7.22-7.32 (m, 3H), 7.32-7.42 (m, 2.4H), 7.42-7.49 (m, 2.0H), 7.50-7.56 (m, 1.6H), 7.60 (t, *J* 7.6 Hz, 0.4H), 7.63-7.80 (m, 4.6H), 7.82 (d, *J* 8.3 Hz, 0.6H), 7.89 (d, *J* 7.7 Hz, 0.4H), 7.96-8.10 (m, 4H), 8.13 (t, *J* 5.8 Hz, 1H), 9.58 (s, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 18.06, 22.50, 23.07, 28.65, 29.00, 32.74, 34.78, 40.35, 40.43, 41.93, 48.28, 49.26, 50.31, 52.15, 52.23, 52.75, 55.42, 55.95, 115.70 (TFA), 117.66 (TFA), 121.90, 122.33, 125.48, 127.26, 127.79, 128.70, 129.71, 130.06, 130.96, 131.56, 132.29, 133.07, 133.78, 134.75, 135.78, 156.78 (2 × guanidinium group), 158.33 (broad signal, carbonyl group of TFA), 165.75, 166.05, 168.70, 169.62, 171.34, 171.38, 172.56. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{49}H_{77}N_{16}O_7]^+$: 1001.6156, found 1001.6135. RP-HPLC (220 nm): 99% (t_R = 4.7 min, *k* = 5.2). $C_{49}H_{76}N_{16}O_7 \cdot C_{10}H_5F_{15}O_5$ (1001.25 + 570.10).

(*S*)-2-acetamido-5-guanidino-*N*-((*S*)-1-oxo-1-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)amino)propan-2-yl)pentanamide tetrakis(hydrotrifluoroacetate) (77)

Compound **77** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 13 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 9 min) yielded **77** as a white fluffy solid (20 mg, 0.016 mmol, 51%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.16-1.30 (m, 7H), 1.34-1.52 (m, 6H), 1.54-1.60 (m, 2H), 1.62-1.68 (m, 1H), 1.71-1.77 (m, 1H), 1.77-

1.84 (m, 1H), 1.86 (s, 3H), 2.73 (s, 3H), 2.86-3.16 (m, 9H), 3.14-3.44 (m, 7H), 3.56-3.63 (m, 1H), 3.77 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.16-4.23 (m, 2H), 4.37-4.46 (m, 1H), 7.06 (brs, 2H), 7.22-7.31 (m, 2.4H), 7.32-7.40 (m, 1.6H), 7.42-7.49 (m, 1.6H), 7.49-7.56 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.68-7.80 (m, 3.4H), 7.82 (d, *J* 8.1 Hz, 0.6H), 7.89 (d, *J* 7.9 Hz, 0.4H), 7.90-7.96 (m, 1H), 8.01 (d, *J* 7.2 Hz, 1H), 8.08 (d, *J* 7.5 Hz, 1H), 9.60 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 18.02, 22.54, 23.05, 23.54, 25.01, 28.64, 28.97, 32.72, 34.75, 35.09, 40.42, 48.28, 49.11, 49.78, 52.31, 52.61, 52.75, 53.20, 55.34, 55.94, 116.63 (q, *J* 298 Hz, TFA), 124.90, 125.49, 126.77, 127.27, 127.72, 128.27, 128.73, 128.98, 129.59, 129.72, 130.03, 130.39, 130.96, 131.57, 131.63, 133.06, 133.10, 133.79, 134.70, 135.78, 139.45, 140.99, 156.82 (guanidinium group), 158.51 (q, *J* 33 Hz, carbonyl group of TFA), 163.69, 164.19, 165.75, 166.05, 169.75, 171.24. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{41}H_{62}N_{11}O_5]^+$: 788.4930, found 788.4931. RP-HPLC (220 nm): 97% (t_R = 4.9 min, *k* = 5.4). $C_{41}H_{61}N_{11}O_5 \cdot C_8H_4F_{12}O_8$ (788.01 + 456.08).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-oxo-1-((2-oxo-2-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)amino)ethyl)amino)propan-2-yl)pentanamide tetrakis(hydrotrifluoroacetate) (78)

Compound **78** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 13 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 9 min) yielded **78** as a white fluffy solid (26 mg, 0.020 mmol, 62%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.45-1.30 (m, 7H), 1.32-1.40 (m, 2H), 1.41-1.52 (m, 4H), 1.55-1.61 (m, 2H), 1.62-1.69 (m, 1H), 1.71-1.77 (m, 1H), 1.77-1.85 (m, 1H), 1.86 (s, 3H), 2.64-2.83 (m, 3H), 2.88-3.05 (m, 5H), 3.05-3.15 (m, 3H), 3.22-3.37 (m, 5H), 3.38-3.53 (m, 2H), 3.55-3.74 (m, 4H), 3.77 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.21-4.26 (m, 2H), 4.36-4.46 (m, 1H), 7.03 (brs, 2H), 7.21-7.31 (m, 2.4H), 7.31-7.41 (m, 1.6H), 7.40-7.49 (m, 1.6H), 7.49-7.54 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.66-7.80 (m, 3.4H), 7.82 (d, *J* 8.1 Hz, 0.6H), 7.85-7.91 (m, 1.4H), 8.07 (d, *J* 7.7 Hz, 1H), 8.11 (d, *J* 6.9 Hz, 1H), 8.15 (t, *J* 6.0 Hz, 1H), 9.59 (s, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.84, 22.73, 23.03, 23.53, 24.96, 28.62, 28.98, 32.70, 34.74, 35.01, 40.41, 42.05, 48.37, 49.04, 49.84, 52.14, 42.59, 52.72, 53.17, 55.27, 55.91, 116.65 (q, *J* 297 Hz, TFA), 121.88, 122.31, 124.88, 125.47, 127.25, 127.70, 128.24, 128.96, 129.57,

129.69, 130.01, 130.37, 130.94, 131.55, 131.60, 133.04, 133.76, 134.67, 135.76, 139.43, 140.97, 156.78 (guanidinium group), 158.45 (q, *J* 33 Hz, carbonyl group of TFA), 163.68, 164.17, 165.74, 166.03, 169.01, 169.59, 171.48, 172.44. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{43}H_{65}N_{13}O_6]^+$: 845.5145, found 845.5148. RP-HPLC (220 nm): 99% (t_R = 4.8 min, *k* = 5.3). $C_{43}H_{64}N_{13}O_6 \cdot C_8H_4F_{12}O_8$ (845.06 + 456.08).

(S)-2-Acetamido-5-amino-N-((S)-1-(((S)-5-guanidino-1-oxo-1-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (79)

Compound **79** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 16 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 9 min) yielded **79** as a white fluffy solid (21 mg, 0.014 mmol, 49%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.17-1-29 (m, 7H), 1.32-1.61 (m, 11H), 1.61-1.70 (m, 2H), 1.71-1.77 (m, 1H), 1.77-1.83 (m, 1H), 1.63 (s, 3H), 2.57-2.70 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak),2.72-2.83 (m, 3H), 2.83-3.05 (m, 6H), 3.05-3.13 (m, 3H), 3.13-3.56 (m, 7H), 3.56-3.64 (m, 1H), 3.77 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.12-4.19 (m, 1H), 4.21-4.28 (m, 2H), 4.35-4.47 (m, 1H), 7.10 (brs, 2H), 7.22-7.32 (m, 2.4H), 7.32-7.41 (m, 1.8H), 7.41-7.49 (m, 1.4H), 7.49-7.58 (m, 1.6H), 7.60 (t, *J* 7.4 Hz, 0.4H), 7.63-7.85 (m, 6.8H), 7.86-8.01 (m, 2.6H), 8.04-8.15 (m, 2H), 9.58 (s, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.92, 22.51, 23.05, 23.61, 28.64, 28.74, 29.08, 32.73, 34.76, 35.29, 38.49, 40.35, 48.32, 49.17, 50.18, 51.88, 52.25, 52.74, 53.18, 55.40, 55.93, 115.90 (TFA), 117.79 (TFA), 121.90, 122.32, 124.90, 125.48, 126.77, 127.27, 127.72, 128.26, 128.73, 129.71, 130.02, 130.38, 130.96, 131.56, 131.62, 133.05, 133.79, 134.69, 135.78, 139.45, 140.99, 156.87 (guanidinium group), 158.46 (q, *J* 33 Hz, carbonyl group of TFA), 164.19, 165.75, 166.05, 169.68, 171.35, 171.41, 173.10. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{46}H_{72}N_{13}O_6]^+$: 902.5723, found 902.5715. RP-HPLC (220 nm): 99% (t_R = 4.3 min, *k* = 4.7). $C_{46}H_{71}N_{13}O_6 \cdot C_{10}H_5F_{15}O_{10}$ (902.16 + 570.10).

(*R*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (80)

Compound **80** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 17 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 9 min) yielded **80** as a white fluffy solid (17 mg, 0.012 mmol, 39%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.13-1.30 (m, 7H); 1.30-1.61 (m, 11H), 1.61-1.66 (m, 1H), 1.67-1.82 (m, 3H); 1.84 (s, 3H), 2.59-2.70 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.71-2.82 (m, 3H), 2.82-3.13 (m, 9H), 3.13-3.52 (m, 7H), 3.55-3.62 (m, 1H), 3.76 (d, *J* 17 Hz, 0.6H), 3.90 (d, *J* 17 Hz, 0.4H), 4.10-4.16 (m, 1H), 4.18 (m, 2H), 4.35-4.46 (m, 1H), 7.09 (brs, 2H), 7.19-7.30 (m, 2.4H), 7.30-7.39 (m, 1.8H), 7.40-7.48 (m, 1.4H), 7.48-7.56 (m, 1.6H), 7.59 (t, *J* 7.6 Hz, 0.4H), 7.66-7.86 (m, 7.8H), 7.88 (d, *J* 7.6 Hz, 0.6H), 7.91 (d, *J* 7.7 Hz, 1H), 8.16-8.22 (m, 1H), 8.37-8.44 (m, 1H), 9.59 (s, 1H), 10.72 (s, 0.4H), 10.77 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.87, 22.35, 23.06, 23.55, 25.16, 28.61, 28.76, 32.73, 34.77, 38.45, 40.36, 48.45, 49.25, 50.31, 52.14, 52.40, 52.73, 53.19, 55.36, 55.93, 115.72 (TFA), 117.68 (TFA), 121.90, 122.32, 124.90, 125.48, 127.26, 127.71, 128.25, 128.73, 129.71, 130.02, 130.38, 130.96, 131.61, 133.05, 134.69, 135.78, 139.45, 140.98, 156.82 (guanidinium group), 158.39 (q, *J* 33 Hz, carbonyl group of TFA), 164.19, 166.05, 169.94, 171.34, 171.81, 172.12. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{46}H_{72}N_{13}O_6]^+$: 902.5723, found 902.5727. RP-HPLC (220 nm): 99% (t_R = 4.3 min, *k* = 4.7). $C_{46}H_{71}N_{13}O_6 \cdot C_{10}H_5F_{15}O_{10}$ (902.16 + 570.10).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-4-guanidino-1-oxo-1-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)amino)butan-2-yl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (81)

Compound **81** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 20 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 9 min) yielded **81** as a white fluffy solid (23 mg, 0.016 mmol, 51%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.15-1.29

(m, 7H), 1.29-1.47 (m, 3H), 1.48-1.62 (m, 5H), 1.64-1.77 (m, 3H), 1.77-1.83 (m, 1H), 1.83-1.93 (m, 4H), 2.60-2.70 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.72-2.84 (m, 3H), 2.84-3.05 (m, 6H), 3.05-3.54 (m, 10H), 3.57-3.65 (m, 1H), 3.77 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.18-4.26 (m, 3H), 4.37-4.46 (m, 1H), 7.15-7.31 (m, 3.4H), 7.31-7.42 (m, 2.0H), 7.40-7.49 (m, 2H), 7.49-7.57 (m, 1.6H), 7.60 (t, J 7.6 Hz, 0.4H), 7.67-7-85 (m, 7.2H), 7.88 (d, *J* 7.8 Hz, 0.4H), 7.92-7.99 (m, 1H), 8.04 (d, *J* 8.0 Hz, 1H), 8.13 (d, *J* 7.7 Hz, 1H), 8.18 (d, *J* 7.0 Hz, 1H), 9.61 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.58, 22.49, 23.05, 23.51, 23.62, 28.65, 31.35, 32.73, 34.76, 35.32, 37.73, 38.49, 48.57, 49.18, 50.10, 50.39, 52.75, 53.19, 55.26, 55.93, 116.70 (q, *J* 298 Hz, TFA), 121.90, 122.33, 124.91, 125.49, 126.77, 127.27, 128.27, 128.73, 128.99, 129.72, 130.03, 130.39, 130.96, 131.56, 131.63, 133.06, 133.78, 139.46, 141.00, 156.93 (guanidinium group), 158.54 (q, *J* 33 Hz, carbonyl group of TFA), 163.72, 164.19, 165.76, 166.05, 169.81, 171.11, 171.64, 172.38. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{45}H_{70}N_{13}O_6]^+$: 888.5567, found 888.5577. RP-HPLC (220 nm): 99% (t_R = 3.9 min, *k* = 4.1). $C_{45}H_{69}N_{13}O_6 \cdot C_{10}H_5F_{15}O_{10}$ (888.13 + 570.10).

(**S)-2-Acetamido-N1-((S)-1-(((S)-5-amino-1-oxo-1-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-4-guanidino-1-oxobutan-2-yl)succinimide pentakis(hydrotrifluoroacetate) (82)**

Compound **82** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 26 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 11 min) yielded **82** as a white fluffy solid (14 mg, 0.0090 mmol, 30%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^1H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.16-1.30 (m, 4H), 1.30-1.47(m, 3H), 1.47-1.64 (m, 5H), 1.69-1.83 (m, 4H), 1.86 (s, 3H), 1.90-1.98 (m, 1H), 2.46-2.48 (m, 1H, interfering with solvent residual peak), 2.53-2.57 (m, 1H, interfering with solvent residual peak), 2.71-2-83 (m, 3H), 2.83-3.05 (m, 6H), 3.05-3.29 (m, 7H), 3.29-3.57 (m, 5H), 3.59-3.63 (m, 1H), 3.77 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.12-4.17 (m, 1H), 4.22-4.26 (m, 1H), 4.37-4.43 (m, 1H), 4.44-4.50 (m, 1H), 7.02 (s, 1H), 7.05-7.31 (m, 4.6H), 7.32-7.40 (m, 1.8H), 7.41-7.49 (m, 1.4H), 7.49-7.57 (m, 2.4H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.65-7.87 (m, 8H), 7.89 (d, *J* 7.9 Hz, 0.4H), 8.01 (d, *J* 7.9 Hz, 1H), 8.19-8.26 (m, 2H), 9.58 (s, 1H, interfering with the following signal), 9.92 (brs, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 22.50, 23.08, 23.65, 28.41, 28.64, 30.92, 32.74, 35.55, 34.77, 36.76, 37.71, 38.44, 49.37, 50.06, 50.49, 50.62,

52.31, 52.73, 53.18, 55.47, 55.94, 115.82 (TFA), 117.78 (TFA), 122.31, 124.87, 125.48, 127.26, 127.71, 127.76, 129.70, 130.94, 131.62, 133.05, 134.67, 135.78, 141.01, 156.85 (guanidinium group), 158.21 (carbonyl group of TFA), 158.45 (carbonyl group of TFA), 165.76, 165.77, 166.04, 169.02, 169.87, 171.00, 171.24, 171.77, 171.89. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{46}H_{71}N_{14}O_7]^+$: 931.5625, found 931.5624. RP-HPLC (220 nm): 99% (t_R = 4.3 min, *k* = 4.7). $C_{46}H_{70}N_{14}O_7 \cdot C_{10}H_5F_{15}O_{10}$ (931.16 + 570.10).

(*S*)-2-Amino-5-guanidino-*N*-(2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl)piperazin-1-yl)ethyl) pentanamide pentakis(hydrotrifluoroacetate) (85)

Compound **85** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 14 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 10 min) yielded **85** as a white fluffy solid (14 mg, 0.011 mmol, 44%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.14-1.22 (m, 2H), 1.34-1.52 (m, 5H), 1.56-1.65 (m, 2H), 1.67-1.85 (m, 4H), 2.57-2.67 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.81-3.07 (m, 7H), 3.07-3.19 (m, 4H), 3.20-3.53 (m, 6H), 3.57-3.66 (m, 1H), 3.71-3.81 (m, 1.6H), 3.92 (d, *J* 17 Hz, 0.4H), 4.37-4.47 (m, 1H), 7.08-7.32 (m, 3.4H), 7.32-7.41 (m, 2H), 7.41-7.49 (m, 2.2H), 7.49-7.57 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.64-7.80 (m, 2.4H), 7.82 (d, *J* 8.2 Hz, 0.6H), 7.89 (d, *J* 8.0 Hz, 0.4H), 7.97-8.02 (m, 1H), 8.23 (s, 3H), 8.56-8.62 (m, 1H), 9.64 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 1.13, 20.53, 24.11, 28.22, 28.50, 32.01, 32.38, 35.49, 49.15, 50.32, 51.91, 52.50, 52.64, 53.08, 55.24, 55.44, 55.92, 116.64 (q, *J* 297 Hz, TFA), 124.88, 125.47, 126.76, 127.26, 127.71, 128.26, 128.71, 128.96, 129.57, 129,71, 130.01, 130.38, 130.94, 131.55, 131.61, 133.04, 133.10, 133.77, 134.69, 135.77, 139.44, 140.99, 156.93 (guanidinium group), 158.59 (q, *J* 33 Hz, carbonyl group of TFA), 163.68, 164.17, 165.74, 166.04, 168.48. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{35}H_{53}N_{10}O_3]^+$: 661.4297, found 661.4306. RP-HPLC (220 nm): 98% (t_R = 4.3 min, *k* = 4.7). $C_{35}H_{52}N_{10}O_3 \cdot C_{10}H_5F_{15}O_{10}$ (660.87 + 570.10).

(*R*)-2-Amino-5-guanidino-*N*-(2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl)piperazin-1-yl)ethyl) pentanamide pentakis(hydrotrifluoroacetate) (86)

Compound **86** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 14 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 90:10-76:24, t_R = 18 min) yielded **86** as a white fluffy solid (13 mg, 0.011 mmol, 47%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.15-1.23 (m, 2H), 1.32-1.52 (m, 5H), 1.56-1.65 (m, 2H), 1.66-1.72 (m, 2H), 1.73-1.78 (m, 1H), 1.78-1.85 (m, 1H), 2.56-2.72 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.84-3.07 (m, 7H), 3.11 (q, *J* 6.6 Hz, 4H), 3.22-3.54 (m, 6H), 3.57-3.66 (m, 1H), 3.71-3.82 (m, 1.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.36-4.48 (m, 1H), 7.03-7.30 (m, 3.4H), 7.30-7.39 (m, 2H), 7.39-7.50 (m, 2.2H), 7.50-7.58 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.65-7.80 (m, 2.4H), 7.82 (d, *J* 8.0 Hz, 0.6H), 7.89 (d, J 7.9 Hz, 0.4H), 7.96 (t, *J* 5.8 Hz, 1H), 8.17-8.27 (m, 3H), 8.55-8.63 (m, 1H), 9.61 (s, 1H), 10.74 (s, 0.4H), 10.79 (s. 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 1.14, 20.56, 24.13, 28.23, 28.50, 32.02, 32.40, 35.48, 49.14, 50.29, 51.92, 52.64, 53.09, 55.21, 55.43, 55.92, 116.46 (q, *J* 296 Hz, TFA), 121.89, 122.32, 124.89, 125.47, 126.77, 127.26, 127.72, 128.26, 128.72, 128.97, 129.58, 129.71, 130.01, 130.38, 130.95, 131.60, 133.05, 134.69, 135.78, 139.44, 140.98, 156.90 (guanidinium group), 158.52 (q, *J* 34 Hz, carbonyl group of TFA), 163.70. 164.17, 165.75, 166.04, 168.50. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{35}H_{53}N_{10}O_3]^+$: 661.4297, found 661.4299. RP-HPLC (220 nm): 98% (t_R = 4.1 min, *k* = 4.4). $C_{35}H_{52}N_{10}O_3 \cdot C_{10}H_5F_{15}O_{10}$ (660.87 + 570.10).

(*S*)-2-Acetamido-6-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl) piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)hexanamide pentakis(hydrotrifluoroacetate) (87)

Compound **87** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 14 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 9 min) yielded **87** as a white fluffy solid (17 mg, 0.011 mmol, 46%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.14-1.25 (m, 5H), 1.25-1.56 (m, 11H), 1.56-1.69 (m, 4H), 1.72-1.84 (m, 2H), 1.85 (s, 3H), 2.54-2.71

(m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.72-2.83 (m, 3H), 2.83-3.05 (m, 6H), 3.05-3.16 (m, 4H), 3.16-3.57 (m, 6H), 3.57-3.66 (m, 1H), 3.77 (d, *J* 17 Hz, 0.6H), 3.92 (d, *J* 17 Hz, 0.4H), 4.11-4.19 (m, 2H), 4.17 (q, *J* 7.1 Hz, 1H), 4.37-4.47 (m, 1H), 7.13 (brs, 2H), 7.22-7.31 (m, 2.2H), 7.32-7.40 (m, 1.8H), 7.40-7.49 (m, 1.6H), 7.49-7.56 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.67-7.86 (m, 7H), 7.87-7.92 (m, 1.4H), 7.92-7.99 (m, 1H), 8.02-8.10 (m, 2H), 9.63 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.82. 20.65, 22.38, 22.51, 25.00, 26.66, 28.52, 29.01, 31.14, 32.06, 32.41, 35.28, 38.69, 40.35, 48.32, 49.20, 50.15, 52.27, 25.58, 52.65, 53.10, 55.31, 55.92, 116.71 (q, *J* 297 Hz, TFA), 121.89, 122.32, 124.90, 125.48, 126.78, 126.48, 127.27, 127.72, 128.27, 128.72, 128.98, 129.58, 129.71, 130.02, 130.39, 130.95, 131.56, 131.62, 133.05, 133.10, 133.78, 134.70, 135.79, 156.86 (guanidinium group), 158.52 (q, *J* 33 Hz, carbonyl group of TFA), 163.72, 164.19, 165.75, 166.05, 169.71. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{46}H_{72}N_{13}O_6]^+$: 902.5723, found 902.5725. RP-HPLC (220 nm): 98% (t_R = 4.1 min, *k* = 4.4). $C_{46}H_{71}N_{13}O_6 \cdot C_{10}H_5F_{15}O_{10}$ (902.16 + 570.10).

(*S*)-2-Acetamido-6-amino-*N*-((*S*)-5-guanidino-1-oxo-1-((2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl) piperazin-1-yl)ethyl)amino)pentan-2-yl)hexanamide pentakis(hydrotrifluoroacetate) (88)

Compound **88** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 16 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 9 min) yielded **88** as a white fluffy solid (17 mg, 0.012 mmol, 47%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.15-1.23 (m, 2H), 1.23-1.55 (m, 11H), 1.55-1.72 (m, 4H), 1.72-1.78 (m, 1H), 1.78-1.84 (m, 1H), 1.86 (s, 3H), 2.58-2.69 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.70-2.82 (m, 3H), 2.85-3.05 (m, 6H), 3.08 (q, *J* 6.5 Hz, 3H), 3.17-3.57 (m, 7H), 3.57-3.68 (m, 1H), 3.78 (d, *J* 17 Hz, 0.6H), 3.92 (d, *J* 17 Hz, 0.4H), 4.15-4.20 (m, 2H), 4.37-4.48 (m, 1H), 7.12 (brs, 2H), 7.22-7.32 (m, 2.2H), 7.32-7.40 (m, 1.6H), 7.40-7.49 (m, 1.6H), 7.49-7.57 (m, 1.6H), 7.59 (t, *J* 7.5 Hz, 0.4H), 7.68-7.87 (m, 7.2H), 7.89 (d, *J* 7.9 Hz, 0.4H), 7.94-8.04 (m, 2H), 8.08 (d, *J* 7.6 Hz, 1H), 9.62 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 20.62, 22.44, 22.51, 25.06, 26.64, 28.52, 29.04, 31.08, 32.05, 32.42, 35.25, 38.70, 49.25, 50.14, 52.15, 52.52, 52.66, 53.10, 55.31, 55.46, 55.92, 116.63 (q, *J* 297 Hz, TFA),124.93, 125.49,

126.77, 127.28, 127.72, 128.27, 128.72, 128.98, 129.59, 129.72, 130.02, 130.39, 130.96, 131.56, 131.62, 133.05, 133.10, 133.78, 134.70, 135.79, 139.45, 141.00, 156.85 (guanidinium group), 158.52 (q, *J* 33 Hz, carbonyl group of TFA), 163.70, 164.18, 165.76, 166.05, 169.76, 171.45, 171.85. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{43}H_{67}N_{12}O_5]^+$: 831.5352, found 831.5355. RP-HPLC (220 nm): 99% (t_R = 4.1 min, *k* = 4.4). $C_{43}H_{66}N_{12}O_5 \cdot C_{10}H_5F_{15}O_{10}$ (831.08 + 570.10).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (89)

Compound **89** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 16 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 9 min) yielded **89** as a white fluffy solid (16 mg, 0.011 mmol, 42%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.14-1.26 (m, 5H), 1.35-1.71 (m, 13H), 1.72-1.79 (m, 1H), 1.79-1.85 (m, 1H), 1.86 (s, 3H), 2.54-2.65 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.72-2.85 (m, 3H), 2.85-3.06 (m, 6H), 3.08 (q, *J* 6.5 Hz, 3H), 3.15-3.50 (m, 7H), 3.57-3.64 (m, 1H), 3.78 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.13-4.19 (m, 1H), 4.21-4.28 (m, 2H), 4.36-4.47 (m, 1H), 7.11 (brs, 2H), 7.22-7.31 (m, 2.4H), 7.31-7.39 (m, 1.6H), 7.41-7.49 (m, 1.6H), 7.49-7.56 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.66-7.86 (m, 7H), 7.86-7.93 (m, 1.4H), 7.95 (s, 1H), 8.06-8.15 (m, 2H), 9.61 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.94, 22.51, 23.61, 24.99, 28.52, 28.74, 29.10, 32.08, 32.42, 38.49, 48.31, 49.30, 51.87, 52.24, 52.54, 52.67, 53.10, 53.73, 55.94, 113.83 (TFA), 115.80 (TFA), 117.77 (TFA), 121.92, 122.32, 124.93, 125.50, 126.79, 127.27, 127.72, 128.28, 128.75, 129.71, 130.02, 130.40, 130.98, 131.58, 133.05, 133.78, 134.69, 135.78, 156.84 (guanidinium group), 158.33 (q, *J* 33 Hz, carbonyl group of TFA), 165.76, 166.05, 169.67, 171.34, 172.08. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{45}H_{70}N_{13}O_6]^+$: 888.5567, found 888.5569. RP-HPLC (220 nm): 99% (t_R = 4.0 min, *k* = 4.3). $C_{45}H_{69}N_{13}O_6 \cdot C_{10}H_5F_{15}O_{10}$ (888.13 + 570.10).

(*R*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (90)

Compound **90** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 16 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 9 min) yielded **90** as a white fluffy solid (18 mg, 0.012 mmol, 41%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.15-1.26 (m, 5H), 1.35-1.67 (m, 12H), 1.69-1.78 (m, 2H), 1.78-1.83 (m, 1H), 1.85 (s, 3H), 2.55-2.69 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.74-2.83 (m, 3H), 2.86-3.05 (m, 6H), 3.08 (q, *J* 6.5 Hz, 3H), 3.14-3.53 (m, 7H), 3.56-3.64 (m, 1H), 3.77 (d, *J* 17 Hz, 0.6H), 3.92 (d, *J* 17 Hz, 0.4H), 4.12-4.16 (m, 1H), 4.20-4.27 (m, 2H), 4.36-4.47 (m, 1H), 7.12 (brs, 2H), 7.22-7.32 (m, 2.2H), 7.32-7.40 (m, 1.6H), 7.40-7.49 (m, 1.6H), 7.49-7.56 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.67-7.86 (m, 8.2H), 7.89 (d, *J* 7.7 Hz, 0.4H), 7.92 (d, *J* 8.0 Hz, 1H), 8.20 (d, *J* 7.1 Hz, 1H), 8.42 (d, *J* 7.1 Hz, 1H), 9.62 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.85, 20.65, 22.35, 23.56, 25.17, 28.52, 28.60, 28.74, 32.06, 32.41, 35.34, 38.45, 48.48, 49.22, 50.19, 52.17, 52.43, 52.66, 53.11, 55.29, 55.49, 55.94, 116.72 (q, *J* 297 Hz, TFA), 122.33, 124.90, 125.49, 126.77, 127.28, 127.73, 128.27, 128.99, 129.72, 130.03, 130.39, 130.96, 131.57, 131.63, 133.06, 134.70, 135.79, 139.46, 141.00, 135.78, 156.86 (guanidinium group), 158.49 (q, *J* 33 Hz, carbonyl group of TFA), 164.19, 165.76, 166.06, 171.38, 171.83, 172.15. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{45}H_{70}N_{13}O_6]^+$: 888.5567, found 888.5568. RP-HPLC (220 nm): 98% (t_R = 4.0 min, *k* = 4.3). $C_{45}H_{69}N_{13}O_6 \cdot C_{10}H_5F_{15}O_{10}$ (888.13 + 570.10).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-4-guanidino-1-oxo-1-((2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl)piperazin-1-yl)ethyl)amino)butan-2-yl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (91)

Compound **91** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 20 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 9 min) yielded **91** as a white fluffy solid (14 mg, 0.010 mmol, 36%). Ratio of configurational isomers

evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.15-1.21 (m, 2H), 1.21-1.27 (m, 3H), 1.33-1.47 (m, 3H), 1.48-1.63 (m, 5H), 1.64-1.78 (m, 3H), 1.78-1.84 (m, 1H), 1.85-1.91 (m, 4H), 2.57-2.67 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.73-2.82 (m, 2H), 2.83-3.25 (m, 12H), 3.25-3.54 (m, 5H), 3.56-3.64 (m, 1H), 3.77 (d, *J* 17 Hz, 0.6H), 3.92 (d, *J* 17Hz, 0.4H), 4.18-4.25 (m, 3H), 4.37-4.47 (m, 1H), 7.02-7.32 (m, 4H), 7.32-7.41 (m, 2H), 7.41-7.50 (m, 1.6H), 7.50-7.57 (m, 1.6H), 7.60 (t, *J* 7.4 Hz, 0.4H), 7.68-7.84 (m, 7H), 7.89 (d, *J* 7.7 Hz, 0.4H), 7.90-7.96 (m, 1H), 8.03 (d, *J* 7.9 Hz, 1H), 8.12 (d, *J* 7.0 Hz, 1H), 8.18 (d, *J* 7.8 Hz, 1H), 9.60 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.59, 20.64, 22.49, 23.62, 28.52, 28.64, 31.36, 32.07, 32.42, 37.73, 38.49, 48.55, 49.32, 50.25, 50.37, 51.96, 52.65, 53.12, 55.32, 55.92, 116.76 (q, *J* 298 Hz, TFA), 122.90, 125.49, 127.27, 127.72, 128.26, 128.73, 128.98, 129.60, 129.71, 130.02, 130.38, 130.96, 131.61, 133.06, 134.69, 135.78, 140.98, 156.90 (guanidinium group), 158.46 (q, *J* 33 Hz, carbonyl group of TFA), 164.19, 165.75, 166.05, 169.80, 171.06, 171.62, 172.36. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{44}H_{68}N_{13}O_6]^+$: 874.5410, found 874.5403. RP-HPLC (220 nm): 97% (t_R = 3.6 min, *k* = 3.7). $C_{44}H_{67}N_{13}O_6 \cdot C_{10}H_5F_{15}O_{10}$ (874.11 + 570.10).

(*S*)-2-Acetamido-*N*^{1}-((*S*)-1-(((*S*)-5-amino-1-oxo-1-((2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-4-((diaminomethylene)amino)-1-oxobutan-2-yl)succinimide pentakis(hydrotrifluoroacetate) (92)

Compound **92** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 26 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 10 min) yielded **92** as a white fluffy solid (11 mg, 0.0075 mmol, 27%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.11-1.25 (m, 2H), 1.31-1.47 (m, 3 H), 1.47-1.64 (m, 5H), 1.69-1.84 (m, 4H), 1.86 (s, 3H), 1.90-1.99 (m, 1H), 2.52-2.59 (m, 2H, interfering with the solvent residual peak), 2.71-2.81 (m, 3H), 2.82-3.06 (m, 7H), 3.06-3.30 (m, 7H), 3.30-3.51 (m, 4H), 3.56-3.64 (m, 1H), 3.77 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.12-4.17 (m, 1H), 4.21-4.26 (m, 1H), 4.36-4.44 (m, 1H), 4.45-4.50 (m, 1H), 7.02 (s, 1H), 7.04-7.21 (m, 2H), 7.21-7.32 (m, 2.6H), 7.32-7.41 (m, 2H), 7.41-7.49 (m, 1.4H), 7.49-7.57 (m, 2.4H), 7.60 (t, *J* 7.4 Hz, 0.4H), 7.66-7.86 (m, 7.8H), 7.89 (d, *J* 7.5 Hz, 0.4H), 8.01 (d, *J* 7.8 Hz, 1H), 8.18-8.26 (m, 2H), 9.59 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR

(150.9 MHz, DMSO-d_6): δ (ppm) 22.50, 23.65, 28.40, 28.53, 30.92, 32.09, 32.42, 36.75, 37.71, 38.44, 49.42, 50.06, 50.62, 52.31, 53.12, 55.46, 55.94, 115.84 (TFA), 117.81 (TFA), 121.91, 122.32, 124.82, 125.47, 127.26, 127.52, 127.71, 128.26, 128.72, 129.59, 129.70, 130.04, 130.36, 130.95, 131.57, 133.05, 134.68, 135.77, 136.19, 140.97, 156.84 (guanidinium group), 158.34 (q, *J* 32 Hz, carbonyl group of TFA), 165.77, 166.04, 169.87, 171.00, 171.24, 171.77, 171.89. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{45}H_{69}N_{14}O_7]^+$: 917.5468, found 917.5463. RP-HPLC (220 nm): 98% (t_R = 4.1 min, *k* = 4.4). $C_{45}H_{68}N_{14}O_7 \cdot C_{10}H_5F_{15}O_{10}$ (917.13 + 570.10).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*R*)-5-guanidino-1-oxo-1-((2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (93)

Compound **93** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 16 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 11 min) yielded **93** as a white fluffy solid (19 mg, 0.013 mmol, 47%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.13-1.26 (m, 5H), 1.33-1.63 (m, 11H), 1.64-1.78 (m, 3H), 1.78-1.84 (m, 1H), 1.86 (s, 3H), 2.55-2.66 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.73-2.82 (m, 2H), 2.84-3.05 (m, 6H), 3.05-3.17 (m, 4H), 3.18-3.38 (m, 5H), 3.38-3.53 (m, 2H), 3.57-3.64 (m, 1H), 3.78 (d, *J* 17 Hz, 0.6H), 3.92 (d, *J* 17 Hz, 0.4H), 4.16-4.28 (m, 3H), 4.37-4.48 (m, 1H), 7.09 (brs, 2H), 7.23-7.32 (m, 2.4H), 7.32-7.40 (m, 1.6H), 7.40-7.49 (m, 1.6H), 7.50-7.56 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.59-7.83 (m, 7H), 7.89 (d, *J* 7.9 Hz, 0.4H), 7.93-7.99 (m, 1H), 7.08 (d, *J* 8.1 Hz, 1H), 8.11 (d, *J* 7.4 Hz, 2H), 9.60 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 18.10, 22.51, 23.60, 25.09, 28.51, 28.71, 29.05, 32.08, 32.42, 38.47, 40.33, 48.39, 49.27, 51.91, 52.15, 52.65, 53.10, 55.40, 55.93, 113.84 (TFA), 115.90 (TFA), 117.77 (TFA), 121.90, 122.32, 124.90, 125.48, 127.26, 127.72, 128.26, 128.73, 129.71, 130.02, 130.38, 130.96, 131.60, 133.05, 133.78, 134.69, 135.78, 139.46, 140.98, 156.82 (guanidinium group), 158.42 (q, *J* 33 Hz, carbonyl group of TFA), 163.68, 164.18, 165.75, 166.05, 169.71, 171.32, 171.41, 172.13. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{45}H_{70}N_{13}O_6]^+$: 888.5567, found 917.5463. RP-HPLC (220 nm): 99% (t_R = 4.1 min, *k* = 4.4). $C_{45}H_{69}N_{13}O_6 \cdot C_{10}H_5F_{15}O_{10}$ (888.13 + 570.10).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-((2-(((*S*)-5-guanidino-1-oxo-1-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)pentan-2-yl)amino)-2-oxoethyl)amino)-1-oxopropan-2-yl)pentanamide tris(hydrotrifluoroacetate) (94)

Compound **94** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 21 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 10 min) yielded **94** as a white fluffy solid (8.8 mg, 0.0075 mmol, 29%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.22 (d, *J* 7.1 Hz, 3H), 1.36-1.54 (m, 6H), 1.59-1.78 (m, 4H), 1.79-1.85 (m, 1H, interfering with the following signal), 1.86 (s, 3H), 1.88-1.98 (m, 1H), 2.99-3.19 (m, 6H), 3.32-3.43 (m, 1H), 3.58-3.81 (m, 4.6H), 3.93 (d, *J* 15 Hz, 0.4H), 4.17-4.29 (m, 3H), 4.36-4.49 (m, 1H), 7.01 (brs, 4H), 7.22-7.32 (m, 3H), 7.32-7.41 (m, 2.6H), 7.43-7.49 (m, 1.4H), 7.49-7.55 (m, 1.4H), 7.58-7.67 (m, 1.4H), 7.68-7.80 (m, 3.2H), 7.82 (d, *J* 8.0 Hz, 0.6H), 7.89 (d, *J* 7.8 Hz, 0.4H), 7.93 (d, *J* 7.8 Hz, 1H), 8.05 (d, *J* 7.5 Hz, 2H), 8.09-8.17 (m, 2H), 9.80 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 18.08, 22.49, 25.00, 28.48, 29.01, 29.42, 40.36, 40.42, 41.92, 43.84, 48.26, 52.10, 115.88 (TFA), 117.85 (TFA), 121.89, 122.32, 124.88, 125.47, 127.24, 127.68, 128.24, 128.73, 128.98, 129.59, 129.68, 130.06, 130.39, 130.97, 131.57, 133.05, 133.7., 134.68, 135.78, 140.95, 156.75 (2 × guanidinium group), 158.42 (q, *J* 33 Hz, carbonyl group of TFA), 165.23, 165.74, 166.03, 168.51, 169.59, 170.75, 171.31, 172.56. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{39}H_{57}N_{14}O_7]^+$: 833.4529, found 833.4530. RP-HPLC (220 nm): 99% (t_R = 5.7 min, *k* = 6.4). $C_{39}H_{56}N_{14}O_7 \cdot C_6H_3F_9O_6$ (832.97 + 342.06).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide tris(hydrotrifluoroacetate) (95)

Compound **95** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 22 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 8 min) yielded **95** as a white fluffy solid (11 mg, 0.0098 mmol, 32%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.20 (d, *J* 7.1 Hz, 3H),1.37-1.55 (m, 6H), 1.58-1.78 (m, 4H), 1.79-1.84 (m, 1H, interfering with the following signal), 1.86 (s, 3H), 1.88-1.95 (m, 1H), 2.99-3.18 (m, 6H), 3.32-3.40 (m,

1H), 3.74-3.96 (m, 3H), 4.09-4.27 (m, 3H), 4.33-4.52 (m, 1H), 7.07 (brs, 4H), 7.22-7.32 (m, 2.6H), 7.32-7.41 (m, 2.4H), 7.42-7.49 (m, 1.6H), 7.49-7.56 (m, 1.6H), 7.60 (t, *J* 7.6 Hz, 0.4H), 7.65-7.80 (m, 4H), 7.82 (d, *J* 7.9 Hz, 1H), 7.89 (d, *J* 7.7 Hz, 1.4H),7.95-8.18 (m, 3H), 9.81 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-*d*$_6$): δ (ppm) 17.89, 22.50, 25.12, 28.47, 28.88, 29.16, 40.41, 43.78, 48.26, 51.74, 52.26, 52.42, 55.78, 116.05 (TFA), 118.03 (TFA), 121.88, 122.32, 124.87, 125.46, 126.71, 127.24, 127.67, 128.26, 218.72, 128.97, 129.70, 130.02, 130.39, 130.95, 131.58, 133.04, 133.76, 134.70, 135.78, 140.98, 156.80 (2 × guanidinium group), 158.50 (carbonyl group of TFA), 158.71 (carbonyl group of TFA), 165.75, 169.79, 170.80, 171.42, 172.01. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{37}H_{54}N_{13}O_6]^+$: 776.4315, found 776.4316. RP-HPLC (220 nm): 99% (t_R = 4.6 min, *k* = 5.0). $C_{37}H_{53}N_{13}O_6 \cdot C_6H_3F_9O_6$ (775.92 + 342.06).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-oxo-1-((2-oxo-2-((2-oxo-2-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)ethyl) amino)ethyl)amino)propan-2-yl)pentanamide bis(hydrotrifluoroacetate) (96)

Compound **96** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 14 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 12 min) yielded **96** as a white fluffy solid (11 mg, 0.0098 mmol, 32%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-*d*$_6$): δ (ppm) 1.22 (d, *J* 7.1 Hz, 3H), 1.41-1.53 (m, 3H), 1.62-1.77 (m, 3H), 1.85 (s, 4H, interfering with the following signal), 1.89-1.97 (m, 1H), 3.00-3.18 (m, 4H), 3.57-3.81 (m, 7H), 3.83-4.03 (m, 1H), 4.18-4.30 (m, 2H), 4.31-4.50 (m, 1H), 6.95 (brs, 2H), 7.21-7.32 (m, 2H), 7.32-7.41 (m, 1.8H), 7.42-7.49 (m, 1.2H), 7.49-7.55 (m, 1.4H), 7.55-7.65 (m, 1.6H), 7.66-7.79 (m, 2H), 7.82 (d, *J* 7.9 Hz, 1H), 7.87-7.95 (m, 1H), 7.99-8.04 (m, 1H), 8.06 (d, *J* 7.8 Hz, 1H), 8.09 (d, *J* 6.9 Hz, 1H),8.14-8.22 (m, 1H), 9.77 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-*d*$_6$): δ (ppm) 17.94, 22.49, 24.97, 28.56, 29.07, 40.42, 41.84, 42.07, 48.27, 51.76, 52.05, 116.23 (TFA), 118.18 (TFA), 121.87, 122.31, 124.86, 125.45, 127.25, 127.67, 128.29, 128.72, 129.69, 130.39, 130.94, 131.56, 133.04, 134.68, 135.77, 156.73 (guanidinium group), 158.11 (q, *J* 32 Hz, carbonyl group of TFA), 165.74, 166.04, 168.22, 169.56, 171.35, 172.63. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{35}H_{48}N_{11}O_7]^+$: 734.3733, found 734.3730. RP-HPLC (220 nm): 98% (t_R = 4.8 min, *k* = 5.3). $C_{35}H_{47}N_{11}O_7 \cdot C_4H_2F_6O_4$ (733.83 + 228.04).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-oxo-1-((2-oxo-2-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)ethyl) amino)propan-2-yl)pentanamid bis(hydrotrifluoroacetate) (97)

Compound **97** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 14 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 12 min) yielded **97** as a white fluffy solid (8.3 mg, 0.0092 mmol, 27%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.21 (d, *J* 7.1 Hz, 3H), 1.41-1.54 (m, 3H), 1.60-1.79 (m, 3H), 1.85 (s, 4H, interfering with the following signal), 1.90-1.98 (m, 1H), 2.99-3.18 (m, 4H), 3.26-3.34 (m, 1H), 3.55-3.74 (m, 4H), 3.79 (d, *J* 17 Hz, 0.6H), 3.93 (d, *J* 17 Hz, 0.4H), 4.18-4.29 (m, 2H), 4.32-4.50 (m, 1H), 6.95 (brs, 2H), 7.20-7.32 (m, 2.6H), 7.32-7.42 (m, 1.6H), 7.42-7.50 (m, 1.2H), 7.50-7.57 (m, 1.2H), 7.57-7.66 (m,1.4H), 7.66-7.80 (m, 2H), 7.82 (d, *J* 8.0 Hz, 0.6H), 7.84-7.94 (m, 1.4H), 7.99-8.13 (m, 3H), 9.76 (s, 1H), 10.74 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.98, 22.53, 25.00, 28.56, 29.03, 40.46, 41.88, 43.91, 48.40, 51.81, 52.17, 55.78, 116.00 (TFA), 117.98 (TFA), 119.96 (TFA), 121.92, 122.37, 124.94, 125.54, 126.75, 127.28, 127.71, 128.29, 128.79, 129.04, 129.72, 130.41, 130.99, 131.61, 133.10, 134.70, 135.80, 141.00, 156.76 (guanidinium group), 158.26 (q, *J* 32 Hz, carbonyl group of TFA), 165.80, 166.09, 168.29, 169.65, 171.47, 172.41. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{33}H_{45}N_{10}O_6]^+$: 677.3518, found 677.3528. RP-HPLC (220 nm): 97% (t_R = 4.9 min, *k* = 5.4). $C_{33}H_{44}N_{10}O_6 \cdot C_4H_2F_6O_4$ (767.78 + 228.04).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-oxo-2-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide tris(hydrotrifluoroacetate) (98)

Compound **98** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 19 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 11 min) yielded **98** as a white fluffy solid (8.9 mg, 0.0075 mmol, 23%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.22 (d, *J* 7.1 Hz, 3H), 1.41-1.59 (m, 6H), 1.60-1.76 (m, 4H), 1.86 (s, 4H), 1.89-1.98 (m, 1H), 2.98-3.19 (m, 6H), 3.33-3.42 (m, 1H), 3.61-3.73 (m, 4H), 3.84-3.99 (m, 1H), 4.14-4.32 (m, 3H), 4.32-4.53 (m, 1H), 7.02 (brs, 4H), 7.21-7.31 (m, 3H), 7.31-7.41 (m, 2.4H), 7.41-

7.49 (m, 1.6H), 7.49-7.56 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.63-7.80 (m, 4H), 7.82 (d, *J* 8.1, Hz, 0.6H), 7.89 (d, *J* 7.5 Hz, 0.4H), 7.94 (s, 1H), 7.99 (d, *J* 7.4 Hz, 1H), 8.01-8.08 (m, 2H), 8.09 (d, *J* 7.7 Hz, 1H), 9.79 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.86, 22.49, 24.89, 25.00, 28.44, 28.90, 40.38, 40.41, 41.77, 48.26, 52.19, 52.27, 52.35, 116.08 (TFA), 118.07 (TFA), 121.88, 122.32, 124.88, 125.46, 126.71, 127.25, 127.67, 128.26, 128.72, 129.69, 130.04, 130.39, 130.96, 131.56, 133.04, 134.49, 135.78, 156.79 (2 × guanidinium group),158.25 (carbonyl group of TFA), 158.51 (carbonyl group of TFA), 165.77, 166.04, 168.09, 169.09, 169.76, 171.37, 171.46, 172.34. HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{39}H_{58}N_{14}O_7]^{2+}$: 417.2301, found 417.2308. RP-HPLC (220 nm): 98% (t_R = 4.6 min, *k* = 5.1). $C_{39}H_{56}N_{14}O_7 \cdot C_6H_3F_9O_6$ (832.97 + 342.06).

(*S*)-2-acetamido-5-guanidino-*N*-((*S*)-1-(((*R*)-5-guanidino-1-oxo-1-((2-oxo-2-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide tris(hydrotrifluoroacetate) (99)

Compound **99** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 19 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 11 min) yielded **99** as a white fluffy solid (7.4 mg, 0.0063 mmol, 20%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.22 (d, *J* 6.8 Hz, 3H), 1.38-1.58 (m, 6H), 1.58-1.78 (m, 4H), 1.87 (s, 4H, interfering with the following signal), 1.89-1.99 (m, 1H), 2.99-3.16 (m, 6H), 3.30-3.39 (m, 1H), 3.62-3.83 (m, 4.6H), 3.87-3.97 (m, 0.4H), 4.16-4.24 (m, 2H), 4.26-4.33 (m, 1H), 4.34-4.51 (m, 1H), 6.99 (brs, 4.6H), 7.21-7.31 (m, 2.4H), 7.31-7.42 (m, 2.8H), 7.42-7.49 (m, 1.2H), 7.50-7.57 (m, 1.6H), 7.60 (t, *J* 7.4 Hz, 0.4H), 7.63-7.79 (m, 4H), 7.82 (d, *J* 8.3, Hz, 0.8H), 7.89 (d, *J* 7.4 Hz, 1.2H), 8.06 (d, *J* 7.0 Hz, 2H), 8.10-8.18 (m, 2H), 9.78 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 18.09, 22.50, 24.99, 28.84, 28.97, 40.35, 40.39, 41.81, 48.28, 52.18, 52.32, 52.41, 116.09 (TFA), 118.07 (TFA), 121.87, 122.31, 124.46, 124.86, 125.44, 127.24, 127.69, 128.22, 128.73, 129.06, 129.58, 129.68, 130.04, 130.40, 130.95, 131.59, 133.04, 135.78, 156.75 (2 × guanidinium group), 158.25 (carbonyl group of TFA), 158.46 (carbonyl group of TFA),165.75, 166.03, 168.12, 169.98, 171.40, 171.50, 172.43. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{39}H_{57}N_{14}O_7]^+$: 833.4529, found 833.4525. RP-HPLC (220 nm): 98% (t_R = 4.6 min, *k* = 5.1). $C_{39}H_{56}N_{14}O_7 \cdot C_6H_3F_9O_6$ (832.97 + 342.06).

(*S*)-2-Acetamido-6-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(2-oxo-2-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)ethyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)hexanamide pentakis(hydrotrifluoroacetate) (101)

Compound **101** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 15 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 11 min) yielded **101** as a white fluffy solid (6.6 mg, 0.0045 mmol, 19%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.21 (d, *J* 7.1 Hz, 3H), 1.24-1.38 (m, 2H), 1.39-1.56 (m, 6H), 1.59-1.79 (m, 4H), 1.86 (s, 4H), 1.90-1.98 (m, 1H), 2.72-2.80 (m, 3H), 2.87-3.19 (m, 11H), 3.26-3.44 (m, 7H), 3.59-3.67 (m, 1H), 3.75-3.79 (m, 1.6H), 3.92 (d, *J* 17 Hz, 0.4H), 4.11-4.20 (m, 2H), 4.24 (q, *J* 7.1 Hz, 1H), 4.34-4.49 (m, 1H), 7.06 (brs, 2H), 7.21-7.32 (m, 2.4H), 7.32-7.41 (m, 1.6H), 7.42-7.49 (m, 1.4H), 7.49-7.58 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.64-7.81 (m, 6.4H), 7.82 (d, *J* 8.2, Hz, 0.6H), 7.89 (d, *J* 7.7 Hz, 0.4H), 7.96 (d, *J* 7.4 Hz, 1H), 8.02-8.12 (m, 3.2H, interfering with the following signal), 8.13-8.31 (m, 1H), 9.81 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.79, 22.39, 22.52, 25.00, 26.66, 28.44, 28.78, 31.11, 38.70, 40.36, 43.81, 48.28, 49.67, 50.49, 52.42, 52.59, 113.86 (TFA), 115.85 (TFA), 117.85 (TFA), 122.36, 124.07, 124.90, 125.47, 127.26, 127.70, 128.29, 128.78, 129.69, 130.05, 130.97, 131.60, 133.06, 133.78, 134.69, 135.78, 138.75, 140.97, 156.79 (guanidinium group), 158.27 (q, *J* 32 Hz, carbonyl group of TFA), 164.32, 164.78, 165.79, 166.04, 169.76, 171.74, 171.79, 172.66. HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{45}H_{70}N_{14}O_7]^{2+}$: 459.2770, found 459.2775. RP-HPLC (220 nm): 98% (t_R = 4.2 min, *k* = 4.5). $C_{45}H_{68}N_{14}O_7 \cdot C_{10}H_5F_{15}O_{10}$ (917.13 + 570.10).

(*S*)-2-Acetamido-6-amino-*N*-((*S*)-5-guanidino-1-oxo-1-((2-(4-(2-oxo-2-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)ethyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)hexanamide pentakis(hydrotrifluoroacetate) (102)

Compound **102** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 17 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 8 min) yielded **102** as a white fluffy solid (16 mg, 0.011 mmol, 41%). Ratio of

configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.20-1.37 (m, 2H), 1.37-1.57 (m, 6H), 1.58-1.79 (m, 4H), 1.86 (s, 3H), 1.91-1.97 (m, 2H), 2.72-2.80 (m, 2H), 2.81-3.18 (m, 11H), 3.19-3.49 (m, 8H), 3.58-3.67 (m, 1H), 3.67-3.74 (m, 1H), 3.79 (d, *J* 16 Hz, 0.6H), 3.93 (d, *J* 16 Hz, 0.4H), 4.13-4.19 (m, 2H), 4.37-4.47 (m, 1H), 7.11 (brs, 2H), 7.22-7.32 (m, 2.2H), 7.32-7.39 (m, 1.8H), 7.43-7.49 (m, 1.6H), 7.49-7.55 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.67-7.86 (m, 7H), 7.89 (d, *J* 7.8, Hz, 0.4H), 8.02 (d, *J* 7.8 Hz, 1H), 8.09 (d, *J* 7.5 Hz, 1H), 8.11-8.17 (m, 1H), 8.26 (s, 1H), 9.85 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 22.44, 22.49, 25.07, 26.63, 28.42, 28.77, 31.01, 38.68, 40.32, 43.83, 49.74, 50.40, 52.28, 52.71, 54.77, 55.72, 116.66 (q, *J* 298 Hz, TFA), 121.88, 122.33, 124.87, 125.47, 127.24, 127.67, 128.23, 128.73, 128.97, 129.59, 129.69, 130.03, 130.38, 130.95, 131.57, 133.05, 133.77, 134.67, 135.78, 139.42, 156.82 (guanidinium group), 158.43 (q, *J* 33 Hz, carbonyl group of TFA), 163.64, 164.13, 165.73, 166.03, 169.85, 171.72, 171.98. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{42}H_{64}N_{13}O_6]^+$: 846.5097, found 846.5091. RP-HPLC (220 nm): 99% (t_R = 3.9 min, *k* = 4.1). $C_{42}H_{63}N_{13}O_6 \cdot C_{10}H_5F_{15}O_{10}$ (846.05 + 570.10).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(2-oxo-2-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)ethyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (103)

Compound **103** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 16 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 8 min) yielded **103** as a white fluffy solid (24 mg, 0.017 mmol, 49%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.22 (d, *J* 7.2 Hz, 3H), 1.39-1.60 (m, 6H), 1.63-1.81 (m, 4H), 1.86 (s, 3H), 1.90-1.97 (m, 2H), 2.72-2.83 (m, 3H), 2.84-3.24 (m, 12H), 3.24-3.44 (m, 6H), 3.59-3.66 (m, 1H), 3.68-3.75 (m, 1H), 3.78 (d, *J* 17 Hz, 0.6H), 3.92 (d, *J* 17 Hz, 0.4H), 4.10-4.16 (m, 1H), 4.20-4.28 (m, 2H), 4.37-4.48 (m, 1H), 7.13 (brs, 2H), 7.22-7.32 (m, 2H), 7.32-7.40 (m, 2H), 7.42-7.50 (m, 1.6H), 7.50-7.56 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.68-7.76 (m, 1.8H), 7.76-7.86 (m, 5.2H), 7.89 (d, *J* 7.8, Hz, 0.4H), 7.97 (d, *J* 7.6 Hz, 1H), 8.08-8.20 (m, 3H), 8.27 (brs, 1H), 9.87 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.86, 22.52, 23.62, 25.02, 28.43, 28.74, 28.83, 38.49, 40.35, 43.83, 48.34, 49.72, 50.43, 51.93, 52.15, 52.43, 54.82, 55.76,

116.71 (q, *J* 298 Hz, TFA), 124.90, 125.50, 126.73, 127.26, 127.70, 128.26, 128.74, 128.99, 129.62, 129.71, 130.05, 130.41, 130.97, 131.60, 133.06, 133.80, 134.70, 135.80, 139.44, 140.98, 156.88 (guanidinium group), 158.50 (q, *J* 33 Hz, carbonyl group of TFA), 164.14, 164.75, 166.05, 169.75, 171.42, 171.74, 172.28. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{44}H_{67}N_{14}O_7]^+$: 903.5312, found 903.5315. RP-HPLC (220 nm): 99% (t_R = 3.9 min, *k* = 4.1). $C_{44}H_{66}N_{14}O_7 \cdot C_{10}H_5F_{15}O_{10}$ (903.10 + 570.10).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-4-((diaminomethylene)amino)-1-oxo-1-((2-(4-(2-oxo-2-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)ethyl)piperazin-1-yl)ethyl)amino)butan-2-yl)amino)-1-oxopropan-2-yl)pentanamid pentakis(hydrotrifluoroacetate) (104)

Compound **104** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 20 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 8 min) yielded **104** as a white fluffy solid (25 mg, 0.017 mmol, 64%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.24 (d, *J* 7.1 Hz, 3H), 1.48-1.63 (m, 3H), 1.63-1.81 (m, 4H), 1.86 (s, 3H), 1.88-1.99 (m, 3H), 2.69-2.85 (m, 4H), 2.85-3.26 (m, 12H), 3.27-3.47 (m, 5H), 3.59-3.67 (m, 1H), 3.68-3.74 (m, 1H), 3.78 (d, *J* 17 Hz, 0.6H), 3.92 (d, *J* 17 Hz, 0.4H), 4.16-4.25 (m, 3H), 4.37-4.48 (m, 1H), 7.05-7.32 (m, 3.6H), 7.32-7.41 (m, 2.2H), 7.42-7.49 (m, 1.6H), 7.49-7.57 (m, 1.6H), 7.60 (t, *J* 7.8 Hz, 0.4H), 7.68-7.86 (m, 7H), 7.89 (d, *J* 7.7Hz, 0.4H), 8.03-8.33 (m, 5.2H), 9.85 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 22,46, 22.52, 26.65, 28.44, 28.79, 31.03, 38.70, 40.34, 43.85, 49.76, 50.42, 52.30, 52.73, 54.79, 55.74, 116.75 (q, *J* 298 Hz, TFA), 121.90, 122.35, 124.89, 125.49, 126.75, 127.26, 127.69, 128.26, 128.99, 129.62, 129.71, 130.06, 130.40, 130.97, 131.59, 133.07, 133.79, 134.70, 135.80, 139.44, 140.97, 156.84 (guanidinium group), 158.47 (q, *J* 33 Hz, carbonyl group of TFA), 164.15, 166.75, 166.05, 169.87, 171.74, 172.00. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{43}H_{65}N_{14}O_7]^+$: 889.5155, found 889.5162. RP-HPLC (220 nm): 99% (t_R = 3.8 min, *k* = 4.0). $C_{43}H_{64}N_{14}O_7 \cdot C_{10}H_5F_{15}O_{10}$ (889.08 + 570.10).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*R*)-5-guanidino-1-oxo-1-((2-(4-(2-oxo-2-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)ethyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (105)

Compound **105** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 16 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 9 min) yielded **105** as a white fluffy solid (18 mg, 0.012 mmol, 45%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.22 (d, *J* 7.2 Hz, 3H), 1.37-1.44 (m, 1H), 1.44-1.61 (m, 5H), 1.64-1.79 (m, 4H), 1.87 (s, 3H), 1.88-2.02 (m, 2H), 2.72-2.83 (m, 4H), 2.87-3.19 (m, 10H), 3.23-3.51 (m, 7H), 3.57-3.66 (m, 1H), 3.66-3.76 (m, 1H), 3.78 (d, *J* 16 Hz, 0.6H), 3.93 (d, *J* 16 Hz, 0.4H), 4.13-4.19 (m, 1H), 4.20-4.28 (m, 2H), 4.36-4.49 (m, 1H), 7.11 (brs, 2H), 7.22-7.32 (m, 2.4H), 7.32-7.41 (m, 1.6H), 7.41-7.49 (m, 1.6H), 7.49-7.56 (m, 1.6H), 7.60 (t, *J* 7.6 Hz, 0.4H), 7.67-7.87 (m, 7H), 7.89 (d, *J* 7.9, Hz, 0.4H), 8.07-8.18 (m, 4H), 8.25 (brs, 1H), 9.85 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 18.02, 22.51, 23.61, 23.61, 25.12, 28.44, 28.70, 28.80, 38.47, 40.33, 43.81, 48.45, 51.95, 52.45, 54.73, 55.75, 116.73 (q, *J* 299 Hz, TFA), 121.89., 122.33, 124.89, 125.48, 126.73, 127.25, 127.68, 128.25, 128.73, 128.98, 129.59, 129.70, 130.04, 130.40, 131.59, 133.05, 133.79, 134.69, 135.79, 140.97, 156.84 (guanidinium group), 158.43 (q, *J* 32 Hz, carbonyl group of TFA), 165.74, 166.04, 169.78, 171.50, 171.67, 172.27. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{44}H_{67}N_{14}O_7]^+$: 903.5312, found 903.5324. RP-HPLC (220 nm): 99% (t_R = 4.0 min, *k* = 4.2). $C_{44}H_{66}N_{14}O_7 \cdot C_{10}H_5F_{15}O_{10}$ (903.10 + 570.10).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*R*)-4-((diaminomethylene)amino)-1-oxo-1-((2-(4-(2-oxo-2-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)ethyl)piperazin-1-yl)ethyl)amino)butan-2-yl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (106)

Compound **106** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 20 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 10 min) yielded **106** as a white fluffy solid (23 mg, 0.016 mmol, 52%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.24 (d, *J* 7.0 Hz, 3H), 1.49-1.62 (m, 3H), 1.64-1.79 (m, 4H), 1.84-1.90 (m, 4H,

interfering with the following signal), 1.90-1.99 (m, 2H), 2.73-2.83 (m, 3H), 2.84-3.21 (m, 11H), 3.21-3.50 (m, 7H), 3.58-3.66 (m, 1H), 3.67-3.75 (m, 1H), 3.79 (d, *J* 16 Hz, 0.6H), 3.93 (d, *J* 16 Hz, 0.4H), 4.15-4.25 (m, 3H), 4.34-4.51 (m, 1H), 6.99-7.31 (m, 4H), 7.31-7.40 (m, 2H), 7.41-7.50 (m, 1.6H), 7.50-7.56 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.68-7.87 (m, 7H), 7.89 (d, *J* 7.8 Hz, 0.4H), 8.00-8.11 (m, 1H), 8.12-8.21 (m, 2.6H), 8.23 (d, *J* 6.6 Hz, 1.4H), 9.83 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.63, 22.50, 23.62, 28.46, 28.58, 30.78, 37.80, 38.46, 43.83, 48.77, 49.77, 50.32, 52.13, 55.75, 116.88 (q, *J* 299 Hz, TFA), 121.89, 122.33,124.87, 125.48, 126.72, 127.25, 127.68, 128.27, 128.73, 129.01, 129.70, 130.04, 130.40, 130.96, 131.58, 133.05, 133.77, 134.68, 135.78, 140.97, 156.91 (guanidinium group), 158.40 (q, *J* 32 Hz, carbonyl group of TFA), 165.72, 166.04, 169.99, 171.42, 171.89, 172.44. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{43}H_{65}N_{14}O_7]^+$: 889.5155, found 889.5164. RP-HPLC (220 nm): 99% (t_R = 3.9 min, *k* = 4.1). $C_{43}H_{64}N_{14}O_7 \cdot C_{10}H_5F_{15}O_{10}$ (889.08 + 570.10).

(*S*)-2-Acetamido-6-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(3-oxo-3-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)propyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)hexanamide pentakis(hydrotrifluoroacetate) (107)

Compound **107** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 16 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 8 min) yielded **107** as a white fluffy solid (28 mg, 0.018 mmol, 48%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.21 (d, *J* 7.1 Hz, 3H), 1.25-1.38 (m, 2H), 1.39-1.55 (m, 7H), 1.59-1.75 (m, 4H), 1.85 (s, 3H, interfering with the following signal), 1.90-1.97 (m, 1H), 2.59-2.84 (m, 6H, interfering with the ^{13}C satellite of the solvent residual peak), 2.90-3.11 (m, 7H), 3.11-3.54 (m, 10H), 3.56-3.73 (m, 2H), 3.79 (d, *J* 17 Hz, 0.6H), 3.93 (d, *J* 17 Hz, 0.4H), 4.11-4.20 (m, 2H), 4.24 (q, *J* 7.1 Hz, 1H), 4.36-4.50 (m, 1H), 7.12 (brs, 2H), 7.22-7.31 (m, 2.2H), 7.33-7.40 (m, 1.6H), 7.41-7.50 (m, 1.6H), 7.50-7.56 (m, 1.6H), 7.60 (t, *J* 7.4 Hz, 0.4H), 7.68-7.84 (m, 7.2H), 7.87-7.93 (m, 1.4H), 7.97 (s, 1H), 8.04-8.10 (m, 2H), 8.25 (s, 1H), 9.84 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.81, 22.38, 22.51, 25.01, 26.66, 28.52, 28.96, 31.14, 38.69, 40.34, 43.83, 48.30, 49.50, 50.18, 51.73, 52.18, 52.30, 52.58, 55.16, 55.72, 116.73 (q, *J* 299 Hz, TFA), 121.89, 122.33, 124.88, 125.48, 126.74, 127.25, 127.68, 128.25, 128.72, 128.98, 129.60, 129.69, 130.03, 130.39, 130.96, 131.59, 133.04, 133.78, 134.69,

135.79, 136.28, 139.44, 140.96, 156.84 (guanidinium group), 158.47 (q, *J* 33 Hz, carbonyl group of TFA), 165.73, 166.03, 169.72, 171.45, 171.73, 172.22. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{46}H_{71}N_{14}O_7]^+$: 931.5625, found 931.5639. RP-HPLC (220 nm): 99% (t_R = 4.0 min, *k* = 4.3). $C_{46}H_{70}N_{14}O_7 \cdot C_{10}H_5F_{15}O_{10}$ (931.16 + 570.10).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-4-guanidino-1-oxo-1-((2-(4-(3-oxo-3-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)propyl)piperazin-1-yl)ethyl)amino)butan-2-yl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (108)

Compound **108** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 20 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 9 min) yielded **108** as a white fluffy solid (16 mg, 0.011 mmol, 52%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.24 (d, *J* 7.2 Hz, 3H), 1.48-1.62 (m, 3H), 1.63-1.77 (m, 4H), 1.83-1.88 (m, 4H, interfering with the following signal), 1.88-1.99 (m, 2H), 2.55-2.89 (m, 7H, interfering with the ^{13}C satellite of the solvent residual peak), 2.89-3.21 (m, 10H), 3.21-3.58 (m, 6H), 3.58-3.73 (m, 2H), 3.79 (d, *J* 16 Hz, 0.6H), 3.93 (d, *J* 16 Hz, 0.4H), 4.14-4.30 (m, 3H), 4.34-4.50 (m, 1H), 7.03-7.23 (m, 1H), 7.23-7.32 (m, 2.6H), 7.32-7.42 (m, 2.2H), 7.41-7.50 (m, 1.6H), 7.50-7.56 (m, 1.6H), 7.60 (t, J 7.6 Hz, 0.4H), 7.66-7.87 (m, 7.2H), 7.89 (d, *J* 7.8 Hz, 0.4H), 7.98 (s, 1H), 8.05 (d, *J* 7.9 Hz, 1H), 8.13 (d, *J* 7.8 Hz, 1H), 8.19 (d, *J* 6.6 Hz, 1H), 8.22-8.30 (m, 1H), 9.84 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.56, 22.49, 23.62, 28.50, 28.65, 31.30, 35.09, 37.72, 38.48, 43.83, 48.56, 49.53, 50.18, 50.39, 51.73, 51.97, 52.16, 55.11, 55.72, 116.62 (q, *J* 298 Hz, TFA), 121.89, 122.33, 124.88, 125.48, 126.72, 127.25, 127.69, 128.25, 128.73, 128.98, 129.59, 129.70, 130.04, 130.39, 130.96, 131.59, 133.04, 133.78, 134.69, 135.79, 139.44, 140.96, 156.91 (guanidinium group), 158.51 (q, *J* 33 Hz, carbonyl group of TFA), 163.68, 164.14, 165.73, 166.03, 169.81, 171.15, 171.65, 172.40. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{44}H_{67}N_{14}O_7]^+$: 903.5312, found 903.5324. RP-HPLC (220 nm): 98% (t_R = 4.0 min, *k* = 4.3). $C_{44}H_{66}N_{14}O_7 \cdot C_{10}H_5F_{15}O_{10}$ (903.10 + 570.10).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(3-oxo-3-((1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)amino)propyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide pentakis(hydrotrifluoroacetate) (109)

Compound **109** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 15 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 8 min) yielded **109** as a white fluffy solid (26 mg, 0.018 mmol, 52%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.22 (d, *J* 7.1 Hz, 3H), 1.38-1.61 (m, 6H), 1.61-1.77 (m, 4H), 1.86 (s, 3H, interfering with the following signal), 1.88-1.98 (m, 2H), 2.59-2.96 (m, 8H, interfering with the ^{13}C satellite of the solvent residual peak), 2.99-3.21 (m, 9H), 3.21-3.35 (m, 4H), 3.35-3.57 (m, 2H), 3.58-3.73 (m, 2H), 3.79 (d, *J* 17 Hz, 0.6H), 3.93 (d, *J* 16 Hz, 0.4H), 4.12-4.19 (m, 1H), 4.20-4.28 (m, 2H), 4.37-4.48 (m, 1H), 7.13 (brs, 2H), 7.22-7.32 (m, 2.2H), 7.32-7.39 (m, 1.6H), 7.40-7.49 (m, 1.6H), 7.49-7.57 (m, 1.6H), 7.60 (t, *J* 7.6 Hz, 0.4H), 7.66-7.86 (m, 7.2H), 7.89 (d, *J* 7.8, Hz, 0.4H), 7.93 (d, *J* 7.8 Hz, 1H), 8.02 (s, 1H), 8.08-8.14 (m, 2H), 8.19-8.29 (m, 1H), 9.85 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.91, 22.51, 23.61, 25.01, 28.46, 28.74, 29.01, 34.98, 38.49, 40.35, 43.84, 48.32, 49.45, 50.13, 51.89, 52.18, 52.30, 55.10, 55.72, 116.68 (q, *J* 298 Hz, TFA), 125.48, 126.72, 127.25, 127.69, 128.25, 128.73, 128.99, 129.59, 129.70, 130.04, 130.39, 130.96, 131.60, 133.05, 134.69, 135.79, 139.45, 140.97, 156.86 (guanidinium group), 158.51 (q, *J* 33 Hz, carbonyl group of TFA), 164.16, 165.74, 166.03, 169.70, 171.37, 171.49, 172.15. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{45}H_{69}N_{14}O_7]^+$: 917.5468, found 917.5483. RP-HPLC (220 nm): 98% (t_R = 3.7 min, *k* = 3.9). $C_{45}H_{68}N_{14}O_7 \cdot C_{10}H_5F_{15}O_{10}$ (917.13 + 570.10).

(*S*)-2-amino-5-guanidino-*N*-(1-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperidin-4-yl)pentanamide tetrakis(hydrotrifluoroacetate) (110)

Compound **110** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 15 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 8 min) yielded **110** as a white fluffy solid (28 mg, 0.026 mmol, 73%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-

d_6): δ (ppm) 1.17-1.29 (m, 4H), 1.33-1.51 (m, 5H), 1.57-1.63 (m, 2H), 1.64-1.83 (m, 6H), 1.95 (t, *J* 14 Hz, 2H), 2.90-3.05 (m, 5H), 3.06-3.17 (m, 3H), 3.29-3.39 (m, 1.4H), 3.48 (t, *J* 12 Hz, 1.6H), 3.56-3.63 (m, 1H), 3.69-3.73 (m, 1H), 3.76-3.91 (m, 1.6H), 4.01-4.08 (m, 0.4H), 4.35-4.47 (m, 1H), 7.05-7.29 (m, 3.4H), 7.29-7.40 (m, 2H), 7.41-7.49 (m, 1.6H), 7.49-7.56 (m, 1.6H), 7.60 (t, *J* 7.6 Hz, 0.4H), 7.67-7.79 (m, 2H), 7.82 (d, *J* 8.2, Hz, 0.6H), 7.88 (d, *J* 7.7 Hz, 0.4H), 7.92-8.03 (m, 1H), 8.23 (s, 3H), 8.68 (d, *J* 7.3 Hz, 1H), 9.61 (s, 1H), 9.95 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 16.71, 23.08, 23.46, 24.14, 28.26, 28.62, 32.71, 34.74, 44.08, 47.28, 50.54, 50.59, 51.91, 52.60, 52.73, 53.16, 53.53, 55.61, 55.92, 116.06 (TFA), 118.05 (TFA), 121.88, 122.31, 124.88, 125.46, 126.77, 127.27, 127.71, 128.71, 128.98, 129.55, 129.71, 130.39, 131.54, 133.03, 134.69, 135.77, 141.00, 156.90 (guanidinium group), 158.50 (brs, carbonyl group of TFA), 164.15, 165.73, 166.04, 167.84. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{35}H_{52}N_9O_2]^+$: 646.4188, found 646.4181. RP-HPLC (220 nm): 99% (t_R = 4.0 min, *k* = 4.2). $C_{35}H_{51}N_9O_2 \cdot C_8H_4F_{12}O_8$ (645.85 + 456.08).

(*R*)-2-amino-5-guanidino-*N*-(1-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperidin-4-yl)pentanamide tetrakis(hydrotrifluoroacetate) (111)

Compound **111** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 17 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 10 min) yielded **111** as a white fluffy solid (32 mg, 0.029 mmol, 77%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.15-1.30 (m, 4H), 1.32-1.52 (m, 5H), 1.56-1.85 (m, 8H), 1.95 (t, *J* 14 Hz, 2H), 2.87-3.05 (m, 5.4H), 3.08-3.17 (m, 2.6H), 3.29-3.40 (m, 1H), 3.48 (t, *J* 13 Hz, 1.6H), 3.56-3.63 (m, 1H), 3.68-3.73 (m, 1H), 3.77 (d, *J* 16 Hz, 0.6H), 3.80-3.88 (m, 1H), 3.91 (d, *J* 17 Hz, 0.4H), 4.02-4.08 (m, 0.4H), 4.33-4.48 (m, 1H), 7.03-7.31 (m, 3.4H), 7.31-7.41 (m, 2H), 7.41-7.49 (m, 1.6H), 7.49-7.56 (m, 1.6H), 7.60 (t, *J* 7.4 Hz, 0.4H), 7.68-7.80 (m, 2H), 7.82 (d, *J* 8.0, Hz, 0.6H), 7.88 (d, *J* 7.9 Hz, 0.4H), 7.91-8.04 (m, 1H), 8.23 (s, 3H), 8.64-8.71 (m, 1H), 9.61 (s, 1H), 9.94 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 23.08, 23.47, 24.14, 28.26, 28.63, 32.71, 34.74, 44.07, 50.55, 50.59, 51.91, 52.58, 52.72, 53.17, 55.60, 55.93, 116.99 (q, *J* 299 Hz, TFA), 121.88, 122.31, 124.88, 125.47, 126.76, 127.26, 127.71, 128.26, 128.70, 128.96, 129.56, 129.71, 130.01, 130.38, 130.95, 131.54, 131.63, 133.04, 133.76, 134.69, 135.78, 139.46, 140.99, 156.90 (guanidinium group), 158.35 (brs, carbonyl group of TFA), 163.68, 164.20, 165.74, 166.04,

167.84. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{35}H_{52}N_9O_2]^+$: 646.4188, found 646.4181. RP-HPLC (220 nm): 99% (t_R = 4.0 min, *k* = 4.1). $C_{35}H_{51}N_9O_2 \cdot C_8H_4F_{12}O_8$ (645.85 + 456.08).

(*S*)-2-Acetamido-6-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((1-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperidin-4-yl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)hexanamide tetrakis(hydrotrifluoroacetate) (112)

Compound **112** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 17 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 10 min) yielded **112** as a white fluffy solid (21 mg, 0.016 mmol, 51%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.20 (d, *J* 7.0 Hz, 4H, interfering with the following signal), 1.22-1.55 (m, 14H), 1.55-1.69 (m, 6H), 1.72-1.82 (m, 2H), 1.85 (s, 3H), 1.87-1.95 (m, 2H), 2.72-2.81 (m, 2H), 2.87-3.04 (m, 5.4H), 3.05-3.16 (m, 2.6H); 3.29-3.38 (m, 1.4H), 3.42-3.50 (m, 1.6H), 3.56-3.65 (m, 1H), 3.71-3.82 (m, 1.6H), 3.88-3.98 (m, 0.4H), 4.12-4.19 (m, 2H), 4.19-4.26 (m, 1H), 4.35-4.49 (m, 1H), 7.16 (brs, 2H), 7.21-7.32 (m, 2H), 7.32-7.39 (m, 1.6H), 7.41-7.49 (m, 1.4H), 7.49-7.56 (m, 1.6H), 7.50 (t, *J* 7.5 Hz, 0.4H), 7.68-7.90 (m, 8H), 8.00-8.08 (m, 2.6H), 8.08-8.14 (m, 0.4H), 9.63 (s, 1H), 9.89 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.78, 17.86, 22.37, 22.51, 23.07, 23.46, 25.04, 26.66, 28.64, 28.72, 29.25, 31.08, 31.17, 32.70, 34.72, 38.40, 38.67, 40.36, 47.35, 48.27, 48.44, 50.69, 50.74, 52.18, 52.35, 52.57, 52.72, 52.82, 53.17, 55.60, 55.93, 116.01 (TFA), 117.99 (TFA), 121.89, 122.32, 124.88, 125.48, 126.76, 127.27, 127.72, 128.27, 128.71, 128.96, 129.56, 129.72, 130.00, 130.38, 130.94, 13 1.54, 131.64, 133.04, 133.11, 133.77, 134.70, 135.78, 139.48, 141.00, 156.86 (guanidinium group), 158.41 (carbonyl group of TFA), 158.61 (carbonyl group of TFA), 163.74, 164.18, 165.75, 166.04, 169.67, 169.85, 170.79, 171.01, 171.68, 171.86, 172.07, 172.12. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{46}H_{71}N_{12}O_6]^+$: 887.5614, found 887.5614. RP-HPLC (220 nm): 99% (t_R = 4.1 min, *k* = 4.4). $C_{46}H_{70}N_{12}O_6 \cdot C_8H_4F_{12}O_8$ (887.14 + 456.08).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((1-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperidin-4-yl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide tetrakis(hydrotrifluoroacetate) (113)

Compound **113** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 17 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 10 min) yielded **113** as a white fluffy solid (22 mg, 0.017 mmol, 55%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.15-1.29 (m, 7H), 1.33-1.71 (m, 15H), 1.72-1.84 (m, 2H), 1.86 (s, 3H), 1.87-1.95 (m, 2H), 2.71-2.83 (m, 2H), 2.88-3.17 (m, 8H), 3.28-3.39 (m, 1.4H), 3.42-3.51 (m, 1.6H), 3.55-3.64 (m, 1H), 3.71-3.81 (m, 1.6H), 3.89-3.97 (m, 0.4H), 4.13-4.19 (m, 1H), 4.20-4.28 (m, 2H), 4.36-4.49 (m, 1H), 7.13 (brs, 2H), 7.22-7.32 (m, 2H), 7.32-7.40 (m, 1.6H), 7.40-7.49 (m, 1.4H), 7.49-7.56 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.67-7.85 (m, 6.4H), 7.85-7.92 (m, 1.6H), 8.04-8.16 (m, 3H), 9.61 (s, 1H), 9.82 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.92, 17.99, 22.50, 23.08, 23.47, 23.60, 25.03, 28.63, 28.72, 28.78, 29.30, 32.70, 34.73, 38.49, 40.47, 43.87, 48.24, 50.70, 50.74, 51.85, 52.04, 52.18, 52.59, 52.73, 53.17, 55.61, 55.94, 115.93 (TFA), 117.91 (TFA), 121.90, 122.32, 124.89, 125.48, 126.76, 127.27, 127.72, 128.26, 128.72, 128.99, 129.57, 129.71, 130.01, 130.38, 130.95, 131.56, 131.63, 133.04, 133.10, 133.78, 134.69, 135.78, 139.47, 140.99, 156.84 (guanidinium group), 158.45 (q, *J* 32 Hz, carbonyl group of TFA), 163.70, 164.18, 165.75, 166.04, 169.64, 169.78, 170.81, 171.03, 171.29, 171.43, 171.99, 172.04. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{45}H_{69}N_{12}O_6]^+$: 873.5458, found 873.5468. RP-HPLC (220 nm): 99% (t_R = 4.3 min, *k* = 4.7). $C_{45}H_{68}N_{12}O_6 \cdot C_8H_4F_{12}O_8$ (873.12 + 456.08).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-4-((diaminomethylene)amino)-1-oxo-1-((1-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperidin-4-yl)amino)butan-2-yl)amino)-1-oxopropan-2-yl)pentanamide tetrakis(hydrotrifluoroacetate) (114)

Compound **114** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 21 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34, t_R = 10 min) yielded **114** as a white fluffy solid (22 mg, 0.017 mmol, 55%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-

d_6): δ (ppm) 1.58-1.29 (m, 7H), 1.32-1.47 (m, 3H), 1.48-1.84 (m, 12H), 1.86 (s, 3H), 1.88-1.97 (m, 2H), 2.71-2.85 (m, 2H), 2.90-3.18 (m, 8H), 3.34 (d, *J* 11 Hz, 1.4H), 3.47 (d, *J* 11 Hz, 1.6H), 3.55-3.64 (m, 1H), 7.72-3.81 (m, 1.6H), 3.88-3.98 (m, 0.4H), 4.17-4.26 (m, 3H), 4.36-4.47 (m, 1H), 7.07-7.32 (m, 3.6H), 7.32-7.40 (m, 1.8H), 7.40-7.49 (m, 1.6H), 7.49-7.56 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.68-7.76 (m, 2.6H), 7.76-7.85 (m, 4H), 7.88 (d, *J* 7.7, Hz, 0.4H), 8.01 (d, *J* 7.8 Hz, 1H), 8.04 (d, *J* 7.8 Hz, 1H), 8.12 (d, *J* 7.0 Hz, 1H), 8.16 (d, *J* 7.8 Hz, 1H), 9.60 (s, 1H), 9.79 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.60, 17.64, 22.49, 23.08, 23.47, 23.61, 28.68, 31.56, 32.69, 34.73, 37.75, 38.48, 44.01, 48.48, 50.35, 50.73, 51.95, 52.18, 52.60, 52.73, 53.18, 55.62, 55.92, 116.70 (q, *J* 299 Hz, TFA), 121.89, 122.32, 124.89, 125.47, 126.76, 127.26, 127.72, 128.26, 128.72, 128.98, 129.71, 130.01, 130.38, 130.95, 131.57, 131.62, 133.05, 133.09, 133.78, 134.69, 135.78, 139.45, 140.99, 156.88 (guanidinium group), 158.46 (q, *J* 32 Hz, carbonyl group of TFA), 164.19, 165.75, 166.04, 169.78, 170.52, 171.58, 172.27. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{44}H_{67}N_{12}O_6]^+$: 859.5301, found 859.5312. RP-HPLC (220 nm): 99% (t_R = 4.3 min, *k* = 4.7). $C_{44}H_{66}N_{12}O_6 \cdot C_8H_4F_{12}O_8$ (859.09 + 456.08).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-1-oxo-1-((2-oxo-2-((2-(4-(2-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)acetyl)piperazin-1-yl)ethyl)amino)ethyl)amino)propan-2-yl)pentanamide tris(hydrotrifluoroacetate) (115)

Compound **115** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 14 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 90: 10-76:24 t_R = 19 min) yielded **115** as a white fluffy solid (8.6 mg, 0.0073 mmol, 34%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.23 (d, *J* 7.0 Hz, 3H), 1.39-1.54 (m, 5H), 1.61-1.70 (m, 1H), 1.77-1.82 (m, 1H), 1.83-1.89 (m, 5H), 2.31 (d, *J* 5.9 Hz, 2H), 2.87-3.02 (m, 3H), 3.02-3.12 (m, 4H), 3.12-3.20 (m, 2H), 3.29-3.39 (m, 2H), 3.40-3.54 (m, 4H), 3.56-3.62 (m, 1.2H), 3.65-3.80 (m, 3.4H), 3.91 (d, *J* 16 Hz, 0.4H), 4.20-4.30 (m, 3H), 4.34-4.48 (m, 1H), 7.01 (brs, 2H), 7.22-7.32 (m, 2H), 7.32-7.40 (m, 2H), 7.40-7.56 (m, 2.6H), 7.58-7.79 (m, 3.4H), 7.82 (d, *J* 8.0 Hz, 0.6H), 7.89 (d, *J* 7.7 Hz, 0.4H), 8.04-8.15 (m, 3H), 8.18 (t, *J* 5.8 Hz, 1H), 9.62 (s, 1H), 10.13 (brs, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.88, 22.50, 24.98, 28.62, 29.00, 29.83, 33.50, 37.43, 40.42, 42.10, 48.32, 50.93, 51.16, 52.16, 52.62, 53.09, 54.72, 55.92, 115.78 (TFA), 117.75 (TFA), 121.87, 122.33, 124.89, 125.49, 126.75, 127.25, 127.71, 128.27, 128.74, 128.98, 129.59, 129.70, 130.03, 130.38, 130.95,

131.57, 131.62, 133.05, 133.80, 134.68, 135.77, 140.97, 156.77 (guanidinium group), 158.20 (carbonyl group of TFA), 158.42 (carbonyl group of TFA), 163.70, 164.19, 165.75, 166.04, 169.32, 169.51, 169.63, 171.48, 172.54. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{41}H_{59}N_{12}O_7]^+$: 831.4624, found 831.4630. RP-HPLC (220 nm): 97% (t_R = 4.6 min, *k* = 5.1). $C_{41}H_{58}N_{12}O_7 \cdot C_8H_4F_{12}O_8$ (830.99 + 342.06).

(*S*)-2-Acetamido-6-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(2-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)acetyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)hexanamide tetrakis(hydrotrifluoroacetate) (116)

Compound **116** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 14 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34 t_R = 8 min) yielded **116** as a white fluffy solid (20 mg, 0.014 mmol, 47%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.21 (d, *J* 7.1 Hz, 3H), 1.25-1.38 (m, 2H), 1.38-1.58 (m, 8H), 1.59-1.71 (m, 2H), 1.76-7.82 (m, 1H), 1.82-1.90 (m, 5H), 2.30 (d, *J* 5.0 Hz, 2H), 2.70-2.81 (m, 2H), 2.82-3.35 (m, 13H), 3.41-3.45 (m, 3H), 3.56-3.62 (m, 1H), 3.76 (d, *J* 17 Hz, 0.6H), 3.90 (d, *J* 16 Hz, 0.4H), 4.03-4.20 (m, 3H), 4.24 (q, *J* 7.1 Hz, 1H), 4.34-4.47 (m, 1H); 7.14 (brs, 2H), 7.22-7.32 (m, 2H), 7.32-7.49 (m, 3H), 7.49-7.57 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.67-7.92 (m, 7H), 7.97 (d, *J* 7.4 Hz, 1H), 8.08 (d, *J* 7.1 Hz, 2H), 8.19 (s, 1H), 9.66 (s, 1H), 10.29 (brs, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.72, 22.39, 22.52, 25.01, 26.66, 28.69, 29.86, 31.12, 33.70, 37.47, 38.17, 38.69, 40.35, 48.34, 51.06, 51.30, 52.48, 52.65, 53.07, 54.69, 55.95, 117.25 (q, *J* 299 Hz, TFA), 121.89, 122.34, 124.90, 125.51, 126.76, 127.28, 128.72, 128.98, 129.59, 129.72, 130.03, 130.39, 130.96, 131.56, 131.66, 133.05, 133.11, 133.80, 134.70, 135.78, 139.49, 141.01, 156.88 (guanidinium group), 158.53 (q, *J* 32 Hz, carbonyl group of TFA), 164.20, 165.77, 166.06, 169.31, 169.80, 171.81, 181.85, 172.43. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{45}H_{68}N_{13}O_7]^+$: 902.5359, found 902.5357. RP-HPLC (220 nm): 99% (t_R = 4.0 min, *k* = 4.3). $C_{45}H_{67}N_{13}O_7 \cdot C_8H_4F_{12}O_8$ (902.12 + 456.08).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(2-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)acetyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide tetrakis(hydrotrifluoroacetate) (117)

Compound **117** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 17 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34 t_R = 8 min) yielded **117** as a white fluffy solid (21 mg, 0.016 mmol, 48%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.22 (d, *J* 7.1 Hz, 3H), 1.38-1.61 (m, 8H), 1.63-1.71 (m, 2H), 1.74-1.91 (m, 6H), 2.30 (d, *J* 5.0 Hz, 2H), 2.73-2.82 (m, 2H), 2.88-3.37 (m, 13H), 3.41-3.45 (m, 3H), 3.57-3.62 (m, 1H), 3.76 (d, *J* 16 Hz, 0.6H), 3.90 (d, *J* 16 Hz, 0.4H), 4.11-4.18 (m, 1H), 4.20-4.30 (m, 3H), 4.33-4.51 (m, 1H), 7.11 (brs, 2H), 7.22-7.31 (m, 2H), 7.32-7.42 (m, 2H), 7.42-7.48 (m, 1H), 7.49-7.57 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.67-7.85 (m, 6.6H), 7.89 (d, *J* 7.6 Hz, 0.4H), 8.00 (d, *J* 7.2 Hz, 1H), 8.08-8.14 (m, 2H), 8.20 (s, 1H), 9.94 (s, 1H), 10.25 (brs, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.84, 22.51, 23.62, 25.00, 28.72, 29.85, 37.47, 38.48, 40.36, 48.31, 51.20, 51.91, 52.44, 53.08, 55.92, 117.05 (q, *J* 299 Hz, TFA), 120.03, 121.89, 122.33, 124.89, 125.50, 126.75, 127.26, 127.71, 128.26, 128.71, 128.98, 129.71, 130.03, 130.38, 130.95, 131.65, 133.04, 133.78, 134.69, 135.77, 139.45, 141.00, 156.84 (guanidinium group), 158.40 (q, *J* 32 Hz, carbonyl group of TFA), 165.76, 166.05, 169.23, 169.74, 171.40, 171.82, 172.31. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{44}H_{66}N_{13}O_7]^+$: 888.5203, found 888.5207. RP-HPLC (220 nm): 99% (t_R = 4.0 min, *k* = 4.3). $C_{44}H_{65}N_{13}O_7 \cdot C_8H_4F_{12}O_8$ (888.09 + 456.08).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-4-guanidino-1-oxo-1-((2-(4-(2-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)acetyl)piperazin-1-yl)ethyl)amino)butan-2-yl)amino)-1-oxopropan-2-yl)pentanamide tetrakis(hydrotrifluoroacetate) (118)

Compound **118** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 21 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34 t_R = 9 min) yielded **118** as a white fluffy solid (19 mg, 0.015 mmol, 53%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.24 (d, *J* 7.1 Hz, 3H), 1.38-1.62 (m, 5H), 1.64-1.76 (m, 2H), 1.76-1.82 (m, 1H),

1.83-1.94 (m, 6H), 2.26-2.35 (m, 2H), 2.71-2.83 (m, 2H), 2.83-3.21 (m, 9H), 3.22-3.67 (m, 8H), 3.76 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 16 Hz, 0.4H), 4.14-5.26 (m, 4H), 4.34-4.49 (m, 1H), 7.05-7.32 (m, 3.4H), 7.32-7.40 (m, 2H), 7.40-7.49 (m, 1.6H), 7.49-7.57 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.68-7.86 (m, 6.6H), 7.89 (d, *J* 7.8 Hz, 0.4H), 8.10 (d, *J* 7.7 Hz, 1H), 8.13 (d, *J* 7.7 Hz, 1H), 8.15-8.24 (m, 2H), 9.64 (s, 1H), 10.28 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.46, 22.48, 23.62, 29.85, 31.05, 37.46, 37.69, 38.47, 48.58, 50.49, 51.03, 51.28, 51.99, 52.62, 53.07, 54.56, 55.92, 116.89 (q, *J* 300 Hz, TFA), 121.88, 122.33, 124.88, 125.49, 126.74, 127.26, 127.71, 128.26, 128.72, 128.98, 129.59, 129.71, 130.02, 130.38, 130.95, 131.56, 131.64, 133.04, 133.78, 134.69, 135.77, 139.46, 140.98, 156.90 (guanidinium group), 158.45 (q, *J* 32 Hz, carbonyl group of TFA),163.69, 164.18, 165.75, 166.04, 169.29, 169.84, 171.50, 171.70, 172.52. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{43}H_{64}N_{13}O_7]^+$: 874.5046, found 874.5058. RP-HPLC (220 nm): 99% (t_R = 3.8 min, *k* = 4.0). $C_{43}H_{63}N_{13}O_7 \cdot C_8H_4F_{12}O_8$ (874.06 + 456.08).

(*S*)-2-Acetamido-6-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propanoyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)hexanamide tetrakis(hydrotrifluoroacetate) (119)

Compound **119** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 17 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34 t_R = 8 min) yielded **119** as a white fluffy solid (21 mg, 0.015 mmol, 56%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.21 (d, J Hz, 3H), 1.26-1.59 (m, 13H), 1.59-1.71 (m, 2H), 1.74-1.81 (m, 1H), 1.83-1.88 (m, 4H), 2.31-2.38 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.72-2.80 (m, 2H), 2.80-3.25 (m, 10H), 3.25-3.39 (m, 3H), 3.36-3.50 (m, 3H), 3.54-3.61 (m, 1H), 3.76 (d, *J* 17 Hz, 0.6H), 3.90 (d, *J* 16 Hz, 0.4H), 4.09-4.20 (m, 3H), 4.24 (t, *J* 7.1 Hz, 1H), 4.33-4.47 (m, 1H), 7.13 (brs, 2H), 7.23-7.31 (m, 2H), 7.31-7.39 (m, 1.6H), 7.41-7.49 (m, 1.4H), 7.49-7.58 (m, 1.6H), 7.60 (t, *J* 7.6 Hz, 0.4H), 7.68-7.85 (m, 6.6H), 7.88 (d, *J* 7.9 Hz, 0.4H), 7.98 (m, 1H), 8.04-8.12 (m, 2H), 8-19 (s, 1H), 9.59 (s, 1H), 10.23 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.72, 22.39, 22.52, 25.01, 26.66, 28.67, 28.78, 30.44, 31.12, 32.39, 33.68, 38.69, 40.36, 48.34, 51.06, 51.32, 52.49, 52.65, 53.16, 54.69, 55.95, 116.98 (q, *J* 299 Hz, TFA), 121.91, 122.33, 124.91, 125.50, 126.78, 127.28, 127.73, 128.28, 128.73, 129.59, 129.72, 130.02, 130.40, 130.95, 131.55, 131.66, 133.05, 133.80, 134.70, 135.78, 141.01, 156.86 (guanidinium

group), 158.47 (q, *J* 32 Hz, carbonyl group of TFA), 164.21, 165.77, 166.06, 169.80, 170.72, 171.80, 171.86, 172.44. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{46}H_{70}N_{13}O_7]^+$: 916.5516, found 916.5510. RP-HPLC (220 nm): 99% (t_R = 4.1 min, *k* = 4.4). $C_{46}H_{69}N_{13}O_7 \cdot C_8H_4F_{12}O_8$ (916.14 + 456.08).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propanoyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide tetrakis(hydrotrifluoroacetate) (120)

Compound **120** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 18 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34 t_R = 11 min) yielded **120** as a white fluffy solid (9.9 mg, 0.0073 mmol, 28%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.22 (d, *J* 7.2 Hz, 3H), 1.35-1.60 (m, 11H), 1.63-1.71 (m, 2H), 1.75-1.81 (m, 1H), 1.81-1.86 (m, 1H, interfering with the following signal), 1.86 (s, 3H), 2.31-2.38 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.73-2.83 (m, 2H), 2.86-3.20 (m, 9H), 3.25-3.57 (m, 7H), 3.57-3.65 (m, 1H), 3.76 (d, *J* 17 Hz, 0.6H), 3.90 (d, *J* 17 Hz, 0.4H), 4.10-4.16 (m, 1H), 4.20-4.30 (m, 3H), 4.35-4.48 (m, 1H), 7.13 (brs, 2H), 7.22-7.32 (m, 2H), 7.32-7.42 (m, 1.6H), 7.41-7.49 (m, 1.4H), 7.49-7.57 (m, 1.6H), 7.60 (t, *J* 7.4 Hz, 0.4H), 7.68-7.86 (m, 6.6H), 7.88 (d, *J* 7.7 Hz, 0.4H), 8.00 (d, *J* 7.4 Hz, 1H), 8.08-8.16 (m, 2H), 8.22 (s, 1H), 9.59 (s, 1H), 10.26 (s, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.82, 22.50, 23.61, 25.00, 28.72, 28.97, 30.43, 32.38, 33.70, 38.47, 40.35, 41.80, 48.32, 51.08, 51.30, 51.91, 52.45, 52.69, 53.14, 51.64, 55.93, 116.94 (q, *J* 299 Hz, TFA), 121.89, 122.31, 124.90, 125.48, 126.75, 127.27, 127.72, 128.71, 129.71, 130.95, 131.55, 133.04, 133.76, 134.71, 135.78, 141.00, 156.86 (guanidinium group), 158.36 (carbonyl group of TFA), 158.57 (carbonyl group of TFA), 165.76, 166.05, 169.73, 170.70, 171.41, 171.84, 172.32. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{45}H_{68}N_{13}O_7]^+$: 902.5359, found 902.5358. RP-HPLC (220 nm): 99% (t_R = 4.2 min, *k* = 4.5). $C_{45}H_{67}N_{13}O_7 \cdot C_8H_4F_{12}O_8$ (902.12 + 456.08).

(S)-2-Acetamido-5-amino-N-((S)-1-(((S)-4-((diaminomethylene)amino)-1-oxo-1-((2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propanoyl)piperazin-1-yl)ethyl)amino)butan-2-yl)amino)-1-oxopropan-2-yl)pentanamide tetrakis(hydrotrifluoroacetate) (121)

Compound **121** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 21 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34 t_R = 10 min) yielded **121** as a white fluffy solid (22 mg, 0.016 mmol, 59%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.24 (d, *J* 7.0 Hz, 3H), 1.32-1.49 (m, 5H), 1.49-1.62 (m, 3H), 1.64-1.82 (m, 3H), 1.82-1.85 (m, 1H, interfering with the following signal), 1.86 (s, 3H), 1.88-1.94 (m, 1H), 2.32-2.38 (m, 2H, interfering with the ^{13}C satellite of the solvent residual peak), 2.70-2.84 (m, 2H), 2.84-3.20 (m, 9H), 3.23-3.56 (m, 7H), 3.56-3.64 (m, 1H), 3.76 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.14-4.29 (m, 4H), 4.34-4.48 (m, 1H), 7.11-7.39 (m, 5H), 7.40-7.49 (m, 2H), 7.49-7.57 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.68-7.85 (m, 6.6H), 7.88 (d, *J* 7.8 Hz, 0.4H), 8.10 (d, *J* 7.7 Hz, 1H), 8.13 (d, *J* 7.7 Hz, 1H), 8.17-8.25 (m, 2H), 9.59 (s, 1H), 10.29 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.45, 22.48, 23.62, 28.62, 28.96, 30.42, 31.04, 32.38, 33.75, 37.70, 38.47, 41.86, 48.59, 50.50, 51.03, 51.27, 52.56, 52.69, 53.14, 54.57, 55.92, 116.95 (q, *J* 299 Hz, TFA), 121.89, 122.32, 124.90, 125.48, 126.76, 127.27, 127.72, 128.26, 128.72, 128.97, 129.71, 130.01, 130.38, 130.95, 131.55, 131.63, 133.04, 133.11, 133.77, 134.69, 135.78, 139.43, 141.00, 156.91 (guanidinium group), 158.49 (q, *J* 32 Hz, carbonyl group of TFA), 165.75, 166.05, 169.85, 170.70, 171.51, 171.71, 172.54. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{44}H_{66}N_{13}O_7]^+$: 888.5203, found 888.5212. RP-HPLC (220 nm): 99% (t_R = 4.0 min, *k* = 4.2). $C_{44}H_{65}N_{13}O_7 \cdot C_8H_4F_{12}O_8$ (888.09 + 456.08).

(S)-2-Acetamido-6-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butanoyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)hexanamide tetrakis(hydrotrifluoroacetate) (122)

Compound **122** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 17 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34 t_R = 9 min) yielded **122** as a white fluffy solid (18 mg, 0.013 mmol, 42%). Ratio of

configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.15-1.25 (m, 5H), 1.25-1.59 (m, 13H), 1.59-1.71 (m, 2H), 1.71-1.78 (m, 1H), 1.78-1.84 (m, 1H), 1.86 (s, 3H), 2.32 (t, *J* 7.3 Hz, 2H), 2.72-2.80 (m, 2H), 2.83-3.19 (m, 9H), 3.24-3.55 (m, 7H), 3.55-3.63 (m, 1H), 3.77 (d, *J* 17 Hz, 0.6H), 3.92 (d, *J* 17 Hz, 0.4H), 4.09-4.19 (m, 3H), 4.24 (q, *J* 7.1 Hz, 1H), 4.36-4.78 (m, 1H), 7.13 (brs, 2H), 7.22-7.32 (m, 2H), 7.32-7.39 (m, 1.6H), 7.40-7.49 (m, 1.6H), 7.49-7.55 (m, 1.4H), 7.60 (t, *J* 7.4 Hz, 0.4H), 7.67-7.86 (m, 6.6H), 7.88 d, *J* 7.8 Hz, 0.4H), 7.98 (d, *J* 7.6 Hz, 1H), 8.07 (d, *J* 7.6 Hz, 2H), 8.16-8.24 (m, 1H), 9.59 (s, 1H), 10.22 (brs, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.71, 21.43, 22.39, 22.51, 25.00, 26.66, 28.66, 31.12, 31.83, 32.76, 33.63, 34.93, 38.07, 38.68, 40.35, 41.84, 48.33, 51.04, 51.27, 52.49, 52.63, 52.78, 53.24, 54.62, 55.94, 116.85 (q, *J* 298 Hz, TFA), 121.89, 122.32, 124.89, 125.48, 126.77, 127.27, 127.72, 128.97, 129.57, 129.71, 130.01, 130.38, 130.95, 131.55, 131.63, 133.05, 134.69, 135.78, 139.47, 141.00, 156.86 (guanidinium group), 158.49 (q, *J* 33 Hz, carbonyl group of TFA), 164.19, 165.75, 166.04, 169.78, 170.75, 171.79, 171.86, 172.43. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{47}H_{72}N_{13}O_7]^+$: 930.5672, found 930.5672. RP-HPLC (220 nm): 98% (t_R = 4.9 min, *k* = 4.5). $C_{47}H_{71}N_{13}O_7 \cdot C_8H_4F_{12}O_8$ (930.17 + 456.08).

(*S*)-2-Acetamido-6-amino-*N*-((*S*)-5-guanidino-1-oxo-1-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butanoyl) piperazin-1-yl)ethyl)amino)pentan-2-yl)hexanamide tetrakis(hydrotrifluoroacetate) (123)

Compound **123** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 18 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34 t_R = 9 min) yielded **123** as a white fluffy solid (10 mg, 0.0078 mmol, 31%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.51-1.22 (m, 2H), 1.26-1.56 (m, 13H), 1.59-1.83 (m, 4H), 1.86 (s, 3H), 2.32 (t, *J* 7.2 Hz, 2H), 2.70-2.81 (m, 2H), 2.83-3.04 (m, 4H),3.04-3.25 (m, 6H), 3.30-3.37 (m, 3H), 3.40-3.45 (m, 3H), 3.55-3.61 (m, 1H), 3.77 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 16 Hz, 0.4H), 4.11-4.24 (m, 3H), 4.35-4.47 (m, 1H), 7.07 (brs, 2H), 7.22-7.31 (m, 2H), 7.32-7.40 (m, 1.6H), 7.41-7.49 (m, 1.4H), 7.49-7.56 (m, 1.4H), 7.60 (t, *J* 7.6 Hz, 0.4H), 7.68-7.92 (m, 7.2H), 8.05 (d, *J* 7.6 Hz, 1H), 8.10 (d, *J* 7.6 Hz, 1H), 8.17-8.23 (m, 1H), 9.57 (s, 1H), 10.19 (brs, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 21.44, 22.46, 22.51, 25.09, 26.65, 28.67, 30.99, 31.85, 32.77, 34.94, 38.10, 38.70, 40.35, 51.07, 51.35, 52.36, 52.75, 53.24, 54.64, 55.95, 116.01 (TFA), 117.99 (TFA), 121.90, 122.32, 124.91,

125.48, 126.77, 127.27, 127.72, 128.73, 129.71, 130.02, 130.37, 130.97, 131.65, 133.06, 134.67, 135.78, 139.44, 141.00, 156.82 (guanidinium group), 158.39 (q, *J* 32 Hz, carbonyl group of TFA), 164.21, 165.75, 169.91, 170.75, 171.86, 172.08. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{44}H_{67}N_{12}O_6]^+$: 859.5301, found 859.5304. RP-HPLC (220 nm): 97% (t_R = 4.9 min, *k* = 4.9). $C_{44}H_{66}N_{12}O_6 \cdot C_8H_4F_{12}O_8$ (859.09 + 456.08).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butanoyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide tetrakis(hydrotrifluoroacetate) (124)

Compound **124** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 16 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34 t_R = 9 min) yielded **124** as a white fluffy solid (19 mg, 0.014 mmol, 44%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.15-1.25 (m, 5H), 1.35-1.61 (m, 11H), 1.63-1.72 (m, 2H), 1.72-1.78 (m, 1H), 1.78-1.84 (m, 1H), 1.87 (s, 3H), 2.32 (t, *J* 7.3 Hz, 2H), 2.72-2.83 (m, 2H), 2.86-3.19 (m, 9H), 3.22-3.54 (m, 7H), 3.56-3.62 (m, 1H), 3.77 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.11-4.16 (m, 1H), 4.19-4.28 (m, 3H), 4.35-4.47 (m, 1H), 7.14 (brs, 2H), 7.23-7.31 (m, 1.8H), 7.31-.7.39 (m, 1.6H), 7.40-7.49 (m, 1.6H), 7.49-7.57 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.68-7.87 (m, 6.6H), 7.88 (d, *J* 7.9 Hz, 0.4H), 8.00 (d, *J* 7.5 Hz, 1H), 8.08-8.16 (m, 2H), 8.19-8.27 (m, 1H), 9.60 (s, 1H), 10.26 (brs, 1H), 10.73 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.81, 21.44, 22.51, 23.61, 25.01, 28.72, 31.84, 32.76, 33.64, 34.94, 38.08, 38.48, 40.35, 41.85, 48.33, 51.05, 51.30, 51.93, 52.47, 52.64, 52.79, 53.23, 54.65, 55.94, 116.94 (q, *J* 299 Hz, TFA), 121.89, 122.32, 124.90, 125.48, 126.77, 127.27, 127.73, 128.27, 128.72, 128.97, 129.72, 130.02, 130.38, 130.95, 131.55, 131.64, 133.05, 133.12, 133.77, 134.70, 135.78, 138.37, 139.48, 141.00, 156.88 (guanidinium group), 158.50 (q, *J* 32 Hz, carbonyl group of TFA), 163.70, 164.20, 165.75, 166.05, 169.74, 171.75, 171.42, 171.87, 172.35. HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{46}H_{71}N_{13}O_7]^{2+}$: 458.7794, found 458.7801. RP-HPLC (220 nm): 98% (t_R = 4.4 min, *k* = 4.8). $C_{46}H_{69}N_{13}O_7 \cdot C_8H_4F_{12}O_8$ (916.14 + 456.08).

(*R*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butanoyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide tetrakis(hydrotrifluoroacetate) (125)

Compound **125** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 17 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34 t_R = 9 min) yielded **125** as a white fluffy solid (20 mg, 0.015 mmol, 52%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.14-1.27 (m, 5H), 1.32-1.60 (m, 11H), 1.61-1.68 (m, 1H), 1.68-1.77 (m, 2H), 1.77-1.84 (m, 1H); 1.85 (s, 3H), 2.28-2.36 (m, 2H); 2.74-2.83 (m, 2H), 2.85-3.20 (m, 9H), 3.25-3.50 (m, 7H), 3.56-3.62 (m, 1H), 3.77 (d, *J* 16 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.10-4.15 (m, 1H), 4.18-4.31 (m, 3H), 4.36-4.47 (m, 1H), 7.15 (brs, 2H), 7.22-7.31 (m, 2H), 7.32-7.40 (m, 1.6H), 7.42-7.49 (m, 1.4H), 7.49-7.56 (m, 1.6H), 7.60 (t, *J* 7.5 Hz, 0.4H), 7.67-7.91 (m, 7H), 7.99 (d, *J* 7.5 Hz, 1H), 8.03-8.09 (m, 1H), 8.20 (d, *J* 7.3 Hz, 1H), 8.42 (d, *J* 7.2 Hz, 1H), 9.61 (s, 1H), 10.27 (brs, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.76, 21.44, 22.36, 23.56, 25.16, 28.46, 28.63, 28.68, 31.84, 32.76, 33.67, 34.94, 38.09, 38.45, 40.36, 41.89, 48.78, 51.05, 51.30, 52.13, 52.61, 52.79, 53.25, 54.59, 55.95, 116.97 (q, *J* 298 Hz, TFA), 121.90, 122.33, 124.91, 125.49, 126.78, 127.28, 128.28, 128.72, 128.97, 129.58, 129.73, 130.02, 130.39, 130.96, 131.56, 131.66, 133.06, 133.79, 134.70, 135.79, 139.48, 141.02, 156.89 (guanidinium group), 158.52 (q, *J* 32 Hz, carbonyl group of TFA), 163.72, 164.20, 165.76, 166.06, 169.97, 170.77, 171.81, 171.86, 172.40. HRMS (ESI): *m/z* $[M+2H]^{2+}$ calcd. for $[C_{46}H_{71}N_{13}O_7]^{2+}$: 458.7794, found 458.7802. RP-HPLC (220 nm): 99% (t_R = 4.4 min, *k* = 4.8). $C_{46}H_{69}N_{13}O_7 \cdot C_8H_4F_{12}O_8$ (916.14 + 456.08).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-4-((diaminomethylene)amino)-1-oxo-1-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butanoyl) piperazin-1-yl)ethyl)amino)butan-2-yl)amino)-1-oxopropan-2-yl)pentanamide tetrakis(hydrotrifluoroacetate) (126)

Compound **126** was prepared according to the general procedure. Purification by preparative HPLC (gradient (protected intermediate: 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 20 min; gradient (product): 0-30 min: 0.1% aq TFA/acetonitrile 85:15-66:34 t_R = 9 min) yielded **126** as a white fluffy solid (21 mg, 0.016 mmol, 59%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-

d_6): δ (ppm) 1.14-1.20 (m, 2H), 1.23 (d, *J* 7.2 Hz, 3H), 1.32-1.49 (m, 5H), 1.49-1.62 (m, 3H), 1.62-1.69 (m, 1H), 1.69-1.77 (m, 2H), 1.77-1.83 (m, 1H), 1.85 (s, 3H), 1.87-1.94 (m, 1H); 2.31 (t, *J* 7.3 Hz, 2H), 2.71-2.82 (m, 2H), 2.82-3.02 (m, 3H), 3.02-3.24 (m, 7H), 3.28-3.54 (m, 6H), 3.54-3.62 (m, 1H), 3.76 (d, *J* 17 Hz, 0.6H), 3.91 (d, *J* 17 Hz, 0.4H), 4.14-4.27 (m, 4H), 4.34-4.47 (m, 1H), 7.05-7.31 (m, 3.4H), 7.31-7.39 (m, 2H), 7.39-7.47 (m, 1.6H), 7.48-7.55 (m, 1.6H), 7.59 (t, *J* 7.5 Hz, 0.4H), 7.66-7.85 (m, 6.6H), 7.88 (d, *J* 7.9 Hz, 0.4H), 8.09 (d, *J* 7.8 Hz, 1H), 8.12 (d, *J* 7.8 Hz, 1H), 8.17-8.24 (m, 2H), 9.58 (s, 1H), 10.26 (brs, 1H), 10.72 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 17.45, 21.43, 22.48, 23.62, 28.63, 31.02, 31.83, 32.76, 33.72, 34.93, 37.70, 38.04, 38.47, 41.82, 48.60, 50.51, 51.03, 51.26, 52.01, 52.65, 52.78, 53.23, 54.53, 55.95, 116.80 (q, *J* 299 Hz, TFA), 121.90, 122.32, 124.90, 125.48, 126.77, 127.26, 127.72, 128.26, 128.72, 129.71, 130.02, 130.38, 130.95, 131.56, 131.63, 133.05, 133.11, 133.78, 134.69, 135.78, 139.46, 141.00, 156.92 (guanidinium group), 158.50 (q, *J* 32 Hz, carbonyl group of TFA),163.71, 164.19, 165.75, 166.04, 169.85, 170.75, 171.53, 171.71, 172.55. HRMS (ESI): *m/z* $[M+H]^+$calcd. for $[C_{45}H_{68}N_{13}O_7]^+$:902.5359, found 902.5369 RP-HPLC (220 nm): 99% (t_R = 4.3 min, *k* = 4.6). $C_{48}H_{67}N_{13}O_7 \cdot C_8H_4F_{12}O_8$ (902.12 + 456.08).

(*S*)-3-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-4-(((*S*)-1-(((*S*)-5-amino-1-oxo-1-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-4-((diaminomethylene)amino)-1-oxobutan-2-yl)amino)-4-oxobutanoic acid tetrakis (hydrotrifluoroacetate) (130)

The coupling of **72** (48 mg, 0.050 mmol) to the side chain and Fmoc-protected peptide **127** (56 mg, 0.050 mmol) was performed according to the procedure used for the syntheses of compounds **53-60**, **63-65**, **73-82**, **85-99**, **101-126**, **130**, **131**, **134**, **135**, **137-139**. The reaction mixture was subjected to preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 19:81, t_R = 26 min) to isolate the side-chain and Fmoc-protected intermediate **128** as a white solid (34 mg, 35%), which was dissolved in TFA/H_2O (95:5 v/v). The mixture was stirred at room temperature for 3 h, CH_2Cl_2 (~20 mL) was added and the volatiles were removed under reduced pressure. This was repeated once. The product was purified by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 48:52, t_R = 17 min) yielding the Fmoc-protected compound **130** as a white solid (23 mg, 89%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, MeOH-d_4): δ (ppm) 1.28-1.40 (m, 4H), 1.41-1.56 (m, 3H), 1.63-1.72 (m, 3H), 1.72-1.81 (m, 2H), 1.85-1.99 (m, 4H), 2.13-2.21 (m, 1H), 2.70 (t, *J* 6.3 Hz, 2.2H), 2.78 (d, *J* 6.3 Hz, 0.6H),

2.81 (d, *J* 6.3 Hz, 0.8H), 2.85-2.97 (m, 6.4H), 2.99-3.09 (m, 4H), 3.20-3.28 (m, 3H), 3.28-3.30 (m, 2H, interfering with the solvent residual peak), 3.38 (t, *J* 6.4 Hz, 2H), 3.40-3.48 (m, 1.8H), 3.68-3.82 (m, 2H), 4.24 (t, *J* 6.8 Hz, 1H), 4.26-4.31 (m, 1H), 4.31-4.39 (m, 2.4H), 4.39-4.47 (m, 2.8H), 7.25-7.29 (m, 0.4H), 7.29-7.36 (m, 3.6H), 7.37-7.42 (m, 2.4H), 7.46-7.51 (m, 1H), 7.51-7.56 (m, 1.2H), 7.60-7.67 (m, 3.4H), 7.69 (t, *J* 7.8 Hz, 0.6H), 7.76 (t, *J* 7.8 Hz, 0.4H), 7.81 (d, *J* 7.6 Hz, 2H), 7.91 (d, *J* 7.9 Hz, 0.6H), 7.98 (d, *J* 7.7 Hz, 0.4H). ^{13}C-NMR (150.9 MHz, MeOH-d_4): δ (ppm) 24.55, 25.12, 25.24, 29.41, 30.41, 31.64, 34.33, 36.17, 36.64, 37.17, 39.36, 40.10, 50.97, 52.61, 52.99, 53.07, 54.38, 57.01, 57.77, 68.28, 121.05, 123.65, 126.16, 126.24, 127.52, 128.25, 128.47, 128.88, 128.92, 130.14, 130.89, 131.74, 132.37, 133.40, 134.59, 137.06, 142.62, 145.13, 158.54, 158.68, 168.84, 173.60, 173.82, 174.48. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{59}H_{78}N_{13}O_9]^+$: 1112.6040, found 1112.6042. $C_{59}H_{77}N_{13}O_9 \cdot C_8H_4F_{12}O_8$ (1112.60 + 456.08).

(*S*)-3-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-4-(((*S*)-1-(((S)-5-amino-1-oxo-1-((2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl)piperazin-1-yl)ethyl)amino)pentan-2-yl)amino)-4-((diaminomethylene)amino)-1-oxobutan-2-yl)amino)-4-oxobutanoic acid tetrakis(hydrotrifluoroacetate) (131)

The coupling of compound **13** (48 mg, 0.050 mmol) to the side chain and Fmoc-protected peptide **127** (56 mg, 0.050 mmol) was performed according to the procedure used for the syntheses of compounds **53-60**, **63-65**, **73-82**, **85-99**, **101-126**, **130**, **131**, **134**, **135**, **137-139**. The reaction mixture was subjected to preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 19:81, t_R = 25 min) to isolate the side-chain and Fmoc-protected intermediate **129** as a white solid (37 mg, 38%). Compound **129** (30 mg, 0.017 mmol) was dissolved in TFA/H_2O (95:5 v/v) and the mixture was stirred at room temperature for 3 h. CH_2Cl_2 (20 mL) was added and the volatiles were removed by evaporation. This was repeated once. The product was purified by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 48:52, t_R = 16 min) yielding the Fmoc-protected compound **131** as a white solid (22 mg, 90%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, MeOH-d_4): δ (ppm) 1.28-1.38 (m, 2H), 1.41-1.61 (m, 3H), 1.65-1.81 (m, 5H), 1.86-2.00 (m, 4H), 2.12-2.22 (m, 1H), 2.68 (t, *J* 6.4 Hz, 2.2H), 2.77 (d, *J* 6.3 Hz, 0.4H), 2.80 (d, *J* 6.2 Hz, 1H), 2.84-2.97 (m, 6.4H), 2.99-3.10 (m, 4H), 3.20-3.27 (m, 3H), 3.28-3.29 (m, 2H, interfering with solvent residual peak), 3.36 (t, *J* 6.4 Hz, 2.4H), 3.41-3.50 (m, 1.4H), 3.70-3.82 (m, 2H), 4.24 (t, *J* 6.8 Hz, 1H), 4.27-4.31 (m, 1H), 4.32-4.38 (m, 2H), 4.39 (s, 0.4H), 4.41-4.47 (m, 2.8H), 7.25-7.36 (m, 4H), 7.37-7.42 (m, 2.4H), 7.46-7.51

(m, 1H), 7.51-7.55 (m, 1.2H), 7.61-7.64 (m, 1H), 7.64-7.67 (m, 2.4H), 7.69 (t, *J* 7.7 Hz, 0.6H), 7.76 (t, *J* 7.7 Hz, 0.4H), 7.81 (d, *J* 7.6 Hz, 2H), 7.91 (d, *J* 7.9 Hz, 0.6H), 7.98 (d, *J* 7.7 Hz, 0.4H). ^{13}C-NMR (150.9 MHz, MeOH-*d*$_4$): δ (ppm) 22.40, 25.12, 29.44, 30.30, 31.64, 36.65, 37.23, 39.36, 40.10, 51.08, 52.72, 52.98, 53.08, 54.37, 57.01, 57.48, 57.76, 68.27, 121.05, 123.10, 123.65, 126.15, 126.23, 127.52, 128.24, 128.47, 128.87, 128.92, 130.15, 131.75, 132.38, 134.59, 142.62, 145.13, 158.67, 173.57, 173.81, 174.35, 174.49. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{58}H_{76}N_{13}O_9]^+$: 1098.5883, found 1098.5884. $C_{58}H_{75}N_{13}O_9 \cdot C_8H_4F_{12}O_8$ (1098.32 + 456.08).

(2*S*,5*S*,12*S*)-12-Amino-2-(2-((diaminomethylene)amino)ethyl)-3,10,13-trioxo-*N*-(2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)-1,4,9-triazacyclotridecane-5-carboxamide pentakis(hydrotrifluoroacetate) (134)

Compound **130** (19 mg, 0.012 mmol) and HOBt (4.6 mg, 0.030 mmol) were dissolved in dry DMF (10 mL) in a 2 mL polypropylene reaction vessels with screw cap. DIPEA (13 µL, 0.072 mmol) and PyBOP (9.4 mg, 0.018 mmol) were added and the mixture was stirred at room temperature for 5 h followed by the addition of 10% aq TFA (72 µL, ca. 0.09 mmol). The Fmoc-protected intermediate **132** was isolated by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 48:52, t_R = 19 min) as a white solid (12 mg, 64%). **132** (8.1 mg, 0.0056 mmol) was dissolved in DMF (0.1 mL), diethylamine (7.5 µL, 0.073 mmol) was added and the mixture was stirred at room temperature for 3 h. 10% aq TFA (73 µL, ca. 0.1 mmol) was added and the mixture was subjected to preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15-57:43, t_R = 10 min) yielding compound **134** as a white solid (7.3 mg, 89 %). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-*d*$_6$): δ (ppm) 1.15-1.28 (m, 5H), 1.30-1.52 (m, 5H), 1.52-1.63 (m, 2H), 1.64-1.83 (m, 4H), 1.87-1.95 (m, 1H), 2.58-2.68 (m, 4H, interfering with the ^{13}C satellite of the solvent residual peak), 2.70-2.78 (m, 1H), 2.86-3.15 (m, 11H), 3.15-3.38 (m, 5H), 3.38-3.56 (m, 2H), 3.56-3.63 (m, 1H), 3.76 (d, *J* 17 Hz, 0.6H), 3.90 (d, *J* 16 Hz, 0.4H), 4.00-4.07 (m, 1H), 4.24 (t, *J* 9.0 Hz, 1H), 4.36-4.47 (m, 2H), 6.91-7.23 (m, 3H), 7.23-7.32 (m, 2.4H), 7.32-7.41 (m, 2H), 7.41-7.49 (m, 1.6H), 7.49-7.58 (m, 1.6H), 7.59 (t, *J* 7.4 Hz, 0.4H), 7.67-7.78 (m, 2H), 7.79-7.76 (m, 1.6H), 7.88 (d, *J* 7.8 Hz, 0.4H), 8.19 (s, 1H), 8.35 (s, 3H), 8.47 (t, *J* 5.9 Hz, 1H), 8.81 (d, *J* 8.1 Hz, 1H), 9.58 (s, 1H), 10.72 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, DMSO-*d*$_6$): δ (ppm) 11.00, 23.06, 23.53, 24.04, 28.53, 28.64, 30.03, 32.73, 34.76, 36.51, 37.50, 37.91, 41.34, 49.20, 49.63, 50.13, 51.27, 52.73, 53.18, 55.39, 55.93, 115.73 (TFA), 117.71 (TFA), 121.89, 122.32, 124.90, 125.48,

127.26, 127.71, 128.27, 128.72, 129.71, 130.02, 130.96, 131.61, 133.04, 134.69, 135.78, 140.98, 156.89 (guanidinium group), 158.42 (q, *J* 32 Hz, carbonyl group of TFA), 164.19, 165.74, 166.04, 167.58, 167.85, 169.17, 170.79. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{44}H_{66}N_{13}O_6]^+$: 872.5254, found 872.5248. RP-HPLC (220 nm): 99% (t_R = 4.1 min, *k* = 4.4). $C_{44}H_{65}N_{13}O_6 \cdot C_{10}H_5F_{15}O_{10}$ (872.09 + 570.10).

(2*S*,5*S*,12*S*)-12-Amino-2-(2-((diaminomethylene)amino)ethyl)-3,10,13-trioxo-*N*-(2-(4-(3-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)propyl)piperazin-1-yl)ethyl)-1,4,9-triazacyclotridecane-5-carboxamide pentakis(hydrotrifluoroacetate) (135)

Compound **135** was prepared from **131** (18 mg, 0.012 mmol) according to the procedure used for the synthesis of compound **134**. HOBt: 4.4 mg, 0.029 mmol., DIPEA: 12 µL, 0.069 mmol. PyBOP: 9.0 mg, 0.017 mmol). After addition of 10% aq TFA (72 µL, ca. 0.09 mmol), the Fmoc-protected intermediate **133** was isolated by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 48:52, t_R = 18 min) as a white solid (11 mg, 63%). Compound **133** (9.0 mg, 0.0063 mmol) was deprotected according to the procedure for **134** (diethylamine: 8.5 µL, 0.082 mmol). 10% aq TFA (82 µL, ca. 0.11 mmol) was added and the product was purified by preparative HPLC (gradient: 0-30 min: 0.1% aq. TFA/acetonitrile 85:15 to 57:43, t_R = 8 min) yielding compound **135** as a white solid (6.1 mg, 68%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.15-1.23 (m, 3H), 1.32-1.52 (m, 5H), 1.55-1.63 (m, 2H), 1.64-1.84 (m, 4H), 1.87-1.95 (m, 1H), 2.56-2.68 (m, 4H), 2.71-2.78 (m, 1H), 2.85-3.15 (m, 11H), 3.15-3.37 (m, 5H), 3.37-3.55 (m, 2H), 3.55-3.65 (m, 1H), 3.78 (d, *J* 16 Hz, 0.6H), 3.92 (d, *J* 16 Hz, 0.4H), 4.00-4.07 (m, 1H), 4.25 (d, *J* 9.0 Hz, 1H), 4.36-4.64 (m, 2H), 6.93-7.23 (m, 3H), 7.23-7.30 (m, 2.2H), 7.30-7.38 (m, 2H), 7.38-7.48 (m, 1.6H), 7.48-7.57 (m, 1.6H), 7.60 (t, *J* 7.4 Hz, 0.4H), 7.67-7.79 (m, 2.2H), 7.80-7.90 (m, 2H), 8.20 (s, 1H), 8.36 (s, 3H), 8.44-8.51 (m, 1H), 8.78-8.85 (m, 1H), 9.60 (s, 1H), 10.74 (s, 0.4H), 10.79 (s, 0.6H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 11.00, 24.03, 28.52, 30.02, 32.06, 32.41, 36.52, 37.50, 37.90, 41.33, 49.26, 49.63, 50.13, 51.26, 52.67, 53.10, 55.38, 55.92, 115.79 (TFA), 117.76 (TFA), 121.87, 122.32, 124.93, 125.47, 127.27, 127.77, 128.24, 128.27, 129.71, 130.04, 130.37, 131.61, 133.05, 134.70, 135.69, 135.78, 136.52, 138.30, 139.46, 141.00, 156.91 (guanidinium group), 158.48 (broad signal, carbonyl group of TFA), 164.26, 165.75, 166.04, 167.58, 167.85, 169.17, 170.77. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{43}H_{64}N_{13}O_6]^+$: 858.5097, found 858.5097. RP-HPLC (220 nm): 99% (t_R = 3.7 min, *k* = 3.9). $C_{43}H_{63}N_{13}O_6 \cdot C_{10}H_5F_{15}O_{10}$ (858.06 + 570.10).

(*S*)-2-Acetamido-6-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-(propylamino)pentan-2-yl)amino)-1-oxopropan-2-yl)hexanamide bis(hydrotrifluoroacetate) (137)

Purification of the side-chain protected peptide was performed by preparative HPLC 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 21 min. The solid was dissolved in TFA/H_2O (95:5 v/v) and the resulting mixture was stirred at room temperature for 3 h. CH_2Cl_2 (20 mL) was added and the volatiles were removed by evaporation. This was repeated once. The residue was dissolved in H_2O and subjected to lyophilization, yielding compound **137** as a white solid (20 mg, 58%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 0.82 (s, 3H), 1.12-1.74 (m, 15H), 1.85 (s, 3H), 2.53-2.64 (m, 2H), 2.91-3.18 (m, 4H), 4.08-4.32 (m, 3H), 7.33 (brs, 4H), 7.64-7.96 (m, 4H), 7.96-8.40 (m, 3H), 10.26 (brs, 1H). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{20}H_{41}N_8O_4]^+$: 457.3245, found 457.3246. $C_{20}H_{40}N_8O_4 \cdot C_4H_2F_6O_4$ (456.59 + 228.04).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-5-guanidino-1-oxo-1-(propylamino)pentan-2-yl)amino)-1-oxopropan-2-yl)pentanamide bis(hydrotrifluoroacetate) (138)

Purification of the side-chain protected peptide was performed by preparative HPLC 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 14 min. The solid was dissolved in TFA/H_2O (95:5 v/v) and the resulting mixture was stirred at room temperature for 3 h. CH_2Cl_2 (20 mL) was added and the volatiles were removed by evaporation. This was repeated once. The residue was dissolved in H_2O and subjected to lyophilization, yielding compound **138** as a white solid (15 mg, 53%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 0.82 (t, *J* 7.3 Hz, 3H), 1.22-1.28 (m, 3H), 1.36-1.49 (m, 4H), 1.52-1.62 (m, 4H), 1.62-1.69 (m, 1H), 1.71-1.79 (m, 1H), 1.86 (s, 3H), 2.75-2.86 (m, 2H), 2.93-3.00 (m, 1H), 3.00-3.13 (m, 3H), 4.13-4.23 (m, 2H), 4.26-4.34 (m, 1H), 6.86-7.50 (m, 4H), 7.75-7.89 (m, 3H), 7.89-8.01 (m, 2H), 8.09 (d, *J* 7.5 Hz, 1H), 8.41 (s, 1H), 10.04 (brs, 1H). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{19}H_{39}N_8O_4]^+$: 443.3089, found 443.3090. $C_{19}H_{38}N_8O_4 \cdot C_4H_2F_6O_4$ (442.57 + 228.04).

(*S*)-2-Acetamido-5-amino-*N*-((*S*)-1-(((*S*)-4-((diaminomethylene)amino)-1-oxo-1-(propylamino)butan-2-yl)amino)-1-oxopropan-2-yl)pentanamide bis(hydrotrifluoroacetate) (139)

Purification of the side-chain protected peptide was performed by preparative HPLC 0-30 min: 0.1% aq TFA/acetonitrile 85:15 to 28:72, t_R = 22 min. The solid was dissolved in

TFA/H_2O (95:5 v/v) and the resulting mixture was stirred at room temperature for 3 h. CH_2Cl_2 (20 mL) was added and the volatiles were removed by evaporation. This was repeated once. The residue was dissolved in H_2O and subjected to lyophilization, yielding compound **139** as a white solid (12 mg, 46%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 0.82 (t, *J* 7.4 Hz, 3H), 1.20-1.29 (m, 3H), 1.36-1.44 (m, 2H), 1.49-1.63 (m, 3H), 1.66-1.80 (m, 2H), 1.86 (s, 3H), 1.87-1.95 (m, 1H), 2.74-2.83 (m, 2H), 2.95-3.06 (m, 2H), 3.06-3.18 (m, 2H), 4.12-4.32 (m, 3H), 7.24 (brs, 4H), 7.67-7.92 (m, 4H), 8.03 (d, *J* 7.5 Hz, 1H), 8.07-8.16 (m, 1H), 8.40 (s, 1H), 10.03 (brs, 1H). HRMS (ESI): *m/z* $[M+H]^+$calcd. for $[C_{18}H_{37}N_8O_4]^+$: 429.2932, found 429.2933. $C_{18}H_{36}N_8O_4 \cdot C_4H_2F_6O_4$ (428.54 + 228.04).

2.4.6. Cell culture

CHO-K9 cells, stably transfected with the DNA of human muscarinic receptors M_1-M_5 (obtained from Missouri S&T cDNA Resource Center; Rolla, MO) were cultured in HAM's F12 medium supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) and G418 (Merck Biochrom, Darmstadt, Germany) (750 µg/mL).

Transfected HEK293T cells, were maintained in DMEM (Thermo Scientific) supplemented with 10% fetal bovine serum (Thermo Scientific), 1 µg/mL puromycin (InvivoGen, Toulouse, France) and 600 µg/mL geneticin (G418) (Merck Biochrom).

2.4.7. Radioligand competition binding assay

Equilibrium competition binding studies with [^{3}H]NMS were performed at intact CHO-hM_xR cells (x = 1-5) at 23 ± 1 °C in white 96-wells plates with clear bottom (Corning Life Science, Tewksbury, MA; Corning cat. No. 3610) using Leibovitz's L-15 medium (Fisher Scientific, Nidderau, Germany) supplemented with 1% BSA (Serva, Heidelberg, Germany) as binding buffer (in the following referred to as L15 medium). Experiments were performed using a previously described protocol for MR binding studies with [^{3}H]NMS,[30] but the total volume per well was 200 µL, i.e. wells were pre-filled with 180 µL of L15 medium followed by the addition of L15 medium (20 µL) containing [^{3}H]NMS (10-fold concentrated), to determine total binding, or pre-filled with 160 µL of L15 medium followed by the addition of L15 medium (20 µL) containing atropine or the compound of interest (10-fold concentrated) and L15 medium (20 µL) containing [^{3}H]NMS (10-fold-concentrated), to determine unspecific binding and the displacing effect of a compound of interest, respectively. The concentrations of

[^{3}H]NMS were 0.2 nM (M_1, M_2, M_3), 0.1 nM (M_4) or 0.3 nM (M_5). Samples were incubated in the dark under gentle shaking for 3 h. Prior to the competition binding experiments, the K_d values of [^{3}H]NMS were determined by saturation binding applying the same conditions (buffer, temperature, incubation time, unspecific binding, etc.). The obtained K_d values, which were in excellent agreement with previously determined K_d values of [^{3}H]NMS[30] are summarized in Table 2.3.

Table 2.3. K_d values of [^{3}H]NMS determined by saturation binding at intact CHO-hM_xR cells (x = 1-5).

MR subtype	pK_d ± SEM / K_d [nM][a]	n	pK_d ± SEM / K_d [nM][b]	n
M_1R	9.77 ± 0.01 / 0.17	4	9.71 ± 0.09 / 0.21	4
M_2R	9.99 ± 0.01 / 0.10	5	10.0 ± 0.05 / 0.094	4
M_3R	9.93± 0.01 / 0.12	5	9.79 ± 0.03 / 0.16	4
M_4R	10.3 ± 0.01 / 0.052	4	10.2 ± 0.04 / 0.062	4
M_5R	9.70 ± 0.02 / 0.20	4	9.75 ± 0.01 / 0.18	4

[a]Mean pK_d values ± SEM and mean K_d-values obtained for [^{3}H]NMS purchased from American Radiolabeled Chemicals Inc. via Hartman Analytics. [b]Mean pK_d values ± SEM and mean K_d-values obtained for [^{3}H]NMS purchased from Novandi. N represents the number of individual experiments each performed in triplicate.

Data from the radioligand ([^{3}H]NMS) competition binding assays were processed as reported previously.[33] pIC_{50} values were converted to pK_i values according to the Cheng-Prusoff equation[43] (logarithmic form).

2.4.8. Mini-G_{si} protein recruitment assay

For construction of the pcDNA3.1 M_2R-NlucC vector, the receptor gene was amplified by PCR without stop codon (fw primer: 5'-gatcaagcttgctagcccaccATGAATAACTCAA-CAAACTCCTC-3'; rv primer: 5'- ctagactcgagccCCTTGTAGCGCCTATGTTC-3' (Eurofins Genomics LLC, Ebersberg, Germany) using pcDNA3.1 M2R (cDNA Recource Center, Bloomsberg, USA) as template. The receptor sequence was then subcloned into the pcDNA3.1 H1R- NlucC replacing the H1R gene by digest with HindIII and XhoI (New England Biolabs) as described previously[38]. The M_2R-NlucC construct was verified by sequencing (Eurofins Genomics LLC). In order to generate HEK293T cells stably expressing NlucN-mini-G_{si} and M_2R-NlucC constructs, the day prior to the transfection, the parental cell line HEK293T NlucN-mini-G_{si}[38] was seeded to a 6-well dish at a density of 0.3 x 10^6 cells/mL in DMEM supplemented with 10% FCS. The cells were transfected with 2 µg of the pcDNA3.1 M_2R-NlucC vector using the XtremeGene HP transfection reagent (Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol. Afterwards,

the cells were cultivated in DMEM supplemented with 10% FCS, 1 µg/mL puromycin and 600 µg/mL G418 for sustained selection pressure.

The day prior to the experiment, HEK293T NlucN-mini-G_{si} / hM_2R-NlucC cells were detached by trypsin treatment (0.05% trypsin, 0.02% EDTA in PBS) and centrifuged (700 g, 5 min). The cells were resuspended in Leibovitz' L-15 medium supplemented with 10 mM HEPES (Serva, Heidelberg, Germany) and 5% FCS. 100.000 cells per well (agonist mode: 80 µL of 1.25 mio cells/mL; antagonist mode: 70 µL of 1.43 mio cells/mL) were seeded to a white flat-bottom 96-well microtiter plate (Merck KGaA, Darmstadt, Germany), followed byincubation at 37°C without CO_2 control (i.e. only atmospheric CO_2) in water-saturated atmosphere overnight.

In the case of the agonist-mode, shortly before the experiment, 10 µL of the substrate furimazine were added to each well and the plate was transferred to a pre-heated (37°C) EnSpire plate reader (Perkin Elmer Inc., Rodgau, Germany). After recording of the basal luminescence for 15 min, 10 µL of the agonist dilutions (10-fold concentrated compared to the final concentration) were added and luminescence traces were recorded for 45 min. The integration timer per well was 0.1 sec, the duration of one plate repeat amounted 30 sec, throughout. The baseline was recorded over 30 repeats and the final measuremt was recorded over 90 or 120 repeats.

When exploring antagonists, the antagonist dilutions (10 µL; 10-fold concentrated compared to the final concentration) were added prior to the agonist and were pre-equilibrated with the cells for 120 minutes. Data were analysed using the GraphPad Prism5 software (San Diego, CA, USA). The absolute luminescence values were corrected for the slight inter-well variability caused by fluctuation in cell density and substrate concentration, as well as for the baseline drift by dividing all data by the solvent control. Area under curves (AUC) of each concentration were normalised to the maximum response of 100 nM or 10 µM iperoxo (100% control) and L-15 (0% control). The normalised responses were plotted against the log concentration of test compound and the data were fitted by a four parameter logistic equation yielding pEC_{50} values of the agonist iperoxo (concentration response curves) or pIC_{50} values of the investigated antagonists (inhibition curves). The latter were converted to pK_b values using the Cheng-Prusoff-equation.[43] Antagonist pK_b values obtained from rightward shifted CRCs of iperoxo were calculated according to the Gaddum equation.[39]

2.4.9. Induced-fit Docking

Induced-fit docking was performed as described previously.[34] Geometries of the ligands were prepared and energetically optimized using the LigPrep module (Schrödinger LLC). The ligands were protonated at, if present, basic nitrogens of piperidine and piperazine rings as well as at the side chains of basic amino acids (primary amine and guanidine groups). In the case of *N,N'*-bisalkylated piperazine moieties, the nitrogen with the shorter distance to the tricyclic headgroup was protonated. For **103** and **109**, this resulted in a formal charge of +4 (protonated structures shown in 2.5. Supplementary Information Figure S15). X-ray crystal structures of the M_1R, M_2R, M_3R, M_4R and M_5R, used for the docking (Table 2.4), were prepared by means of the Protein Preparation Wizard module (Schrödinger LLC). Missing amino acid residues were added using Prime. Receptor amino acid sidechains containing hydrogen bond donors and acceptors were optimized for hydrogen bonding and were modeled in their dominant protonation state at pH 7. For initial docking, two strategies were pursued: Y3.33, Y6.51, and Y7.39 were temporarily mutated to alanine (Gridgen_Recep_Vscale = 0.7), or mutations were omitted (Gridgen_Recep_Vscale = 0.5). Ligands were docked within a box of 46 × 46 × 46 $Å^3$ around the crystallographic binding poses of the co-crystallized ligands. Re-docking was performed in the extended precision mode. Docking poses, corresponding to low score values (preferably the lowest score), being reasonable at the same time, were considered for an evaluation of the binding mode.

Table 2.4. MR crystal structures used for induced-fit docking.

receptor	PDB-ID	co-crystallized ligand	activation state	resolution
M_1R	5CXV	tiotropium	inactive	2.70 Å
M_2R	5ZKB	AF-DX 384	inactive	2.95 Å
M_3R	4U15	tiotropium	inactive	2.60 Å
M_4R	5DSG	tiotropium	inactive	2.60 Å
M_5R	6OL9	tiotropium	inactive	2.54 Å

2.5. Supplementary Information

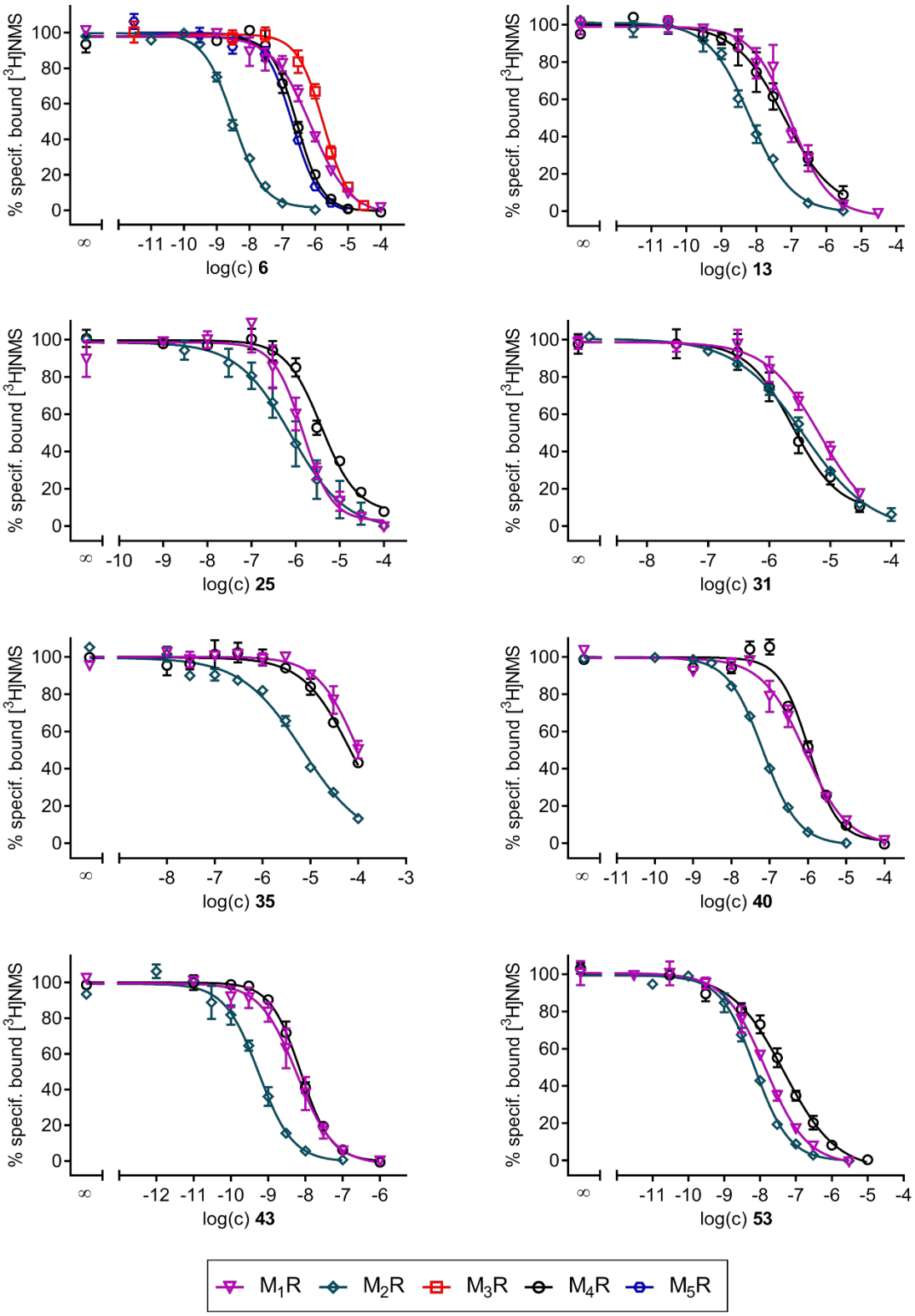


Figure S1. Radioligand displacement curves obtained from competition binding experiments with [^{3}H]NMS (0.2 nM (M_1R, M_2R) or 0.1 nM (M_4R)) and **6**, **13**, **25**, **31**, **35**, **40**, **43**, or **53** at intact CHO-hM_xR cells (x = 1,2,4). In the case of **6**, competition binding experiments were also performed at CHO-hM_3R ([^{3}H]NMS: 0.2 nM) and CHO-hM_5R ([^{3}H]NMS: 0.3 nM) cells, but no curves were obtained.Data represent mean values ± SEM from at least three independent experiments (each performed in triplicate).

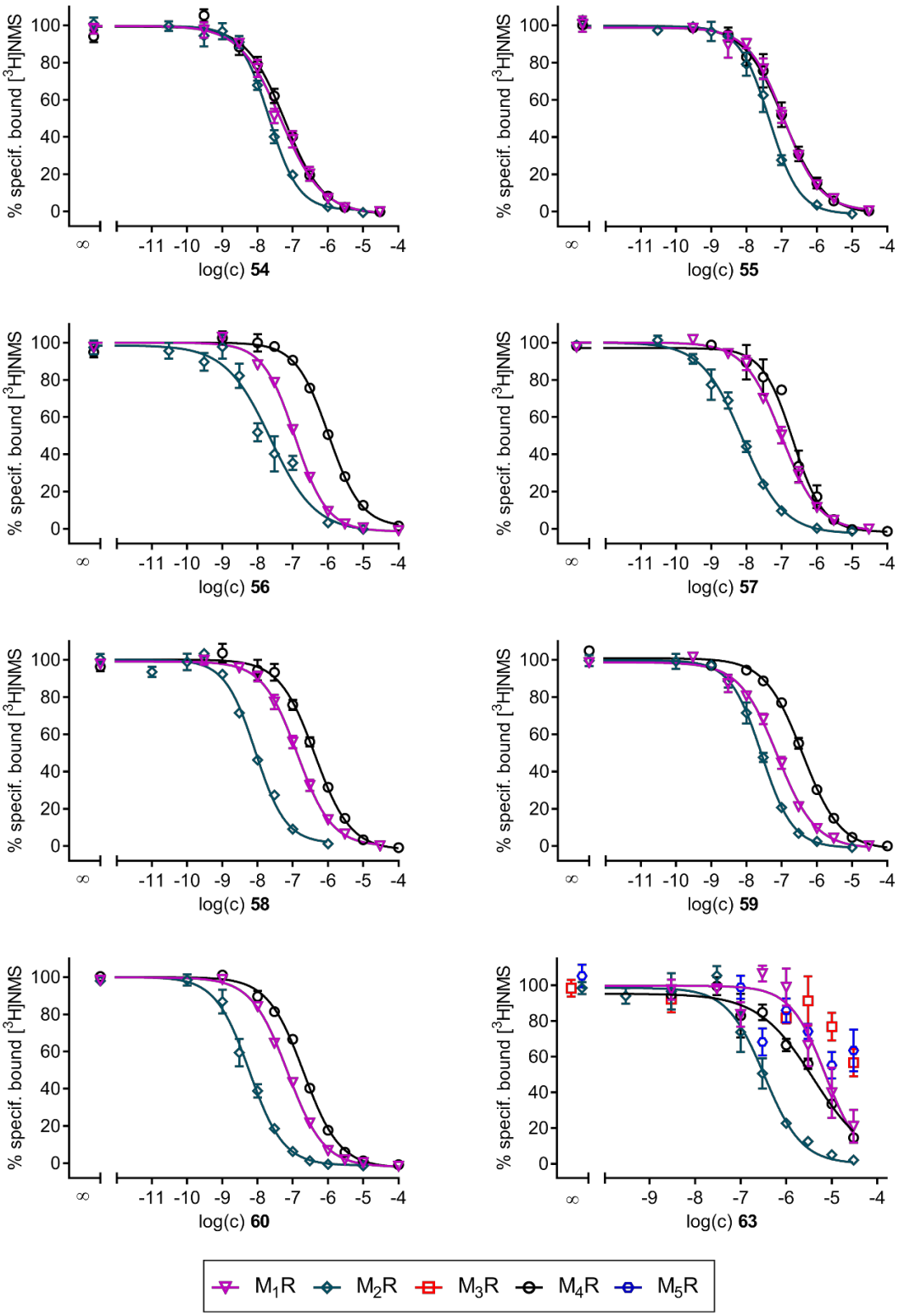


Figure S2. Radioligand displacement curves obtained from competition binding experiments with [3H]NMS (0.2 nM (M_1R, M_2R) or 0.1 nM (M_4R)) and **54**-**60**, or **63** at intact CHO-hM_xR cells (x = 1,2,4). In the case of **63**, competition binding experiments were also performed at CHO-hM_3R ([3H]NMS: 0.2 nM) and CHO-hM_5R ([3H]NMS: 0.3 nM) cells, but no curves were obtained. Data represent mean values ± SEM from two (**63**, M_1R, M_5R) or at least three independent experiments (each performed in triplicate).

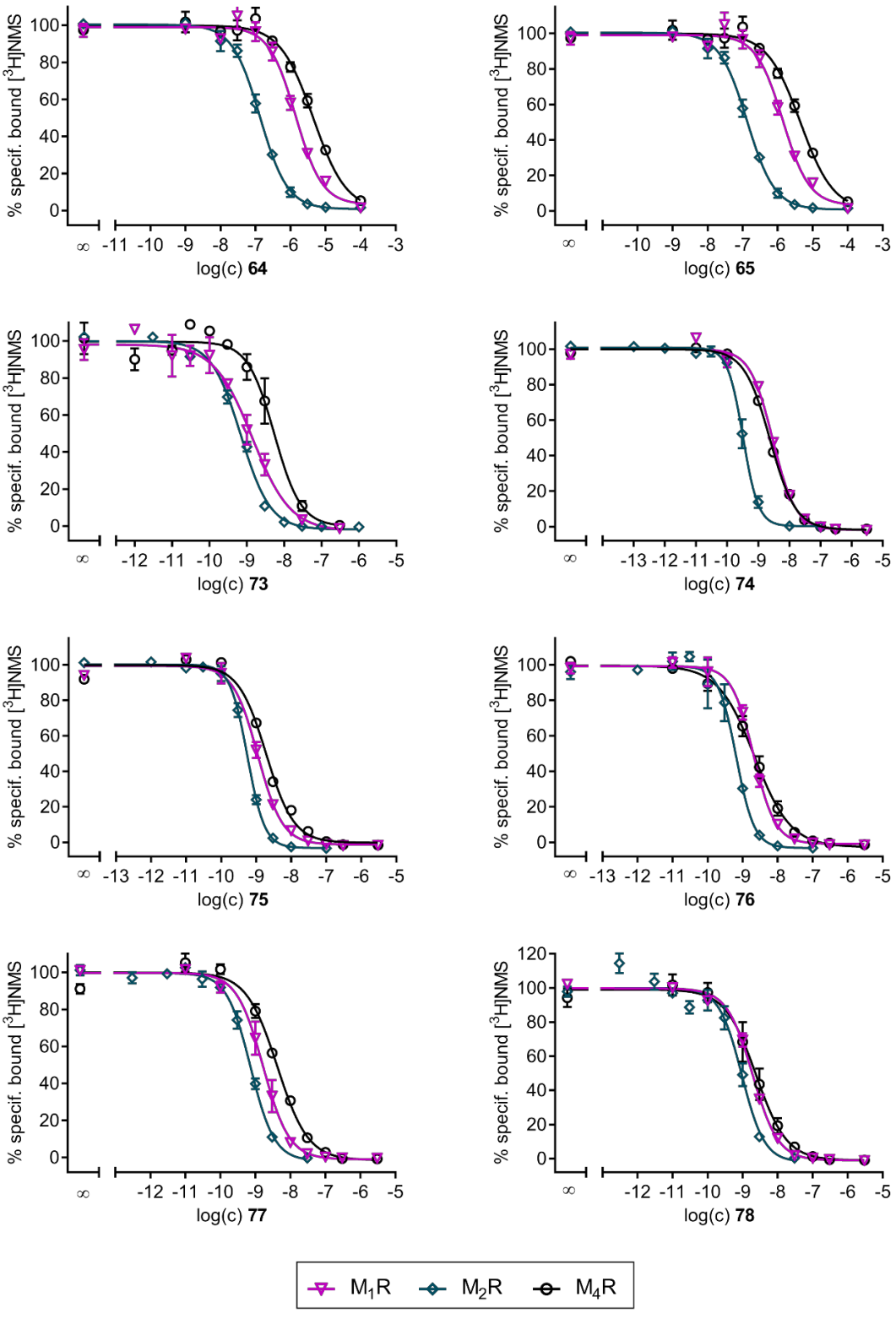


Figure S3. Radioligand displacement curves obtained from competition binding experiments with [^{3}H]NMS (0.2 nM (M_1R, M_2R) or 0.1 nM (M_4R)) and **64**, **65** or **73-78** at intact CHO-hM_xR cells (x = 1,2,4). Data represent mean values ± SEM from at least three independent experiments (each performed in triplicate).

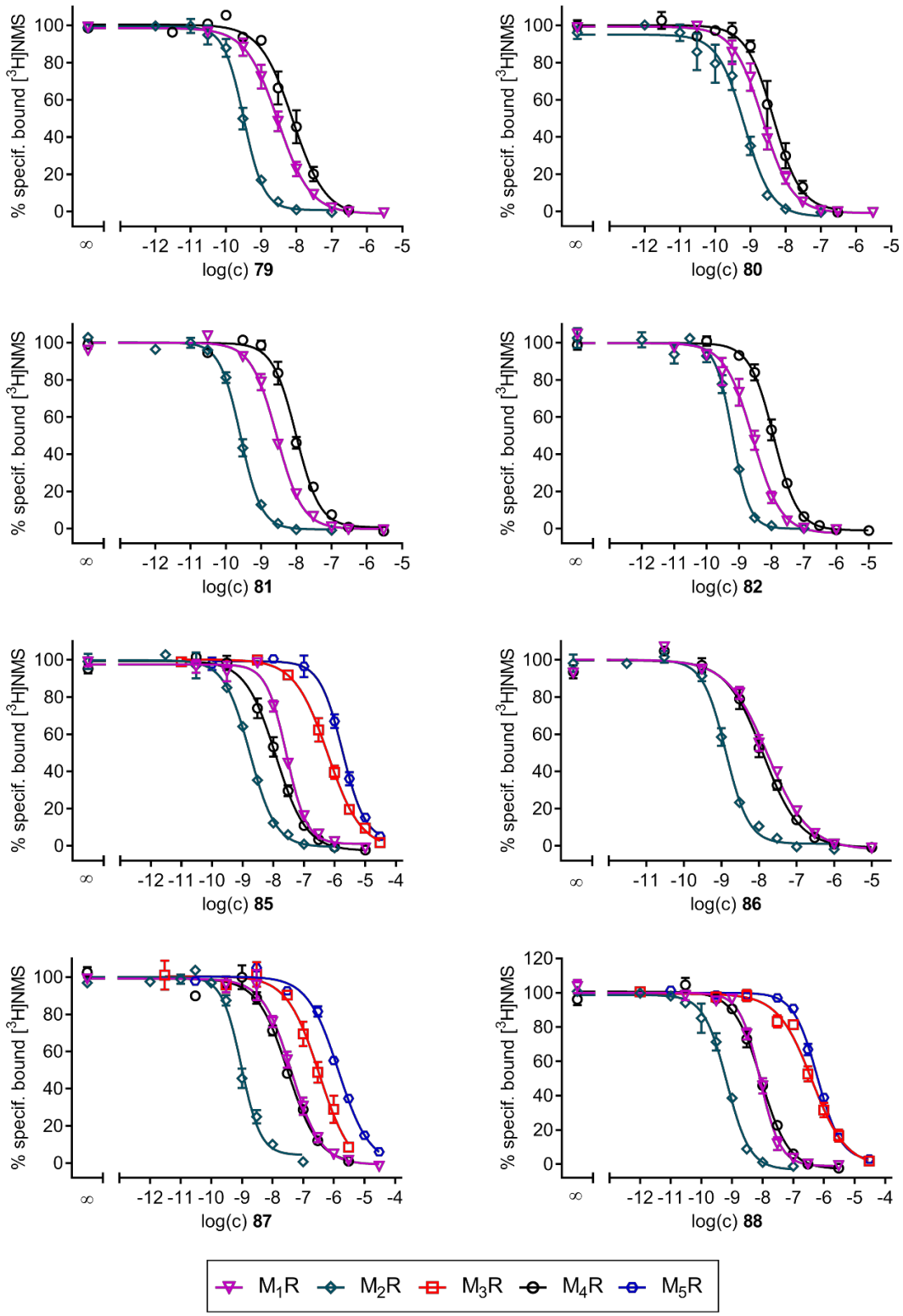


Figure S4. Radioligand displacement curves obtained from competition binding experiments with [^{3}H]NMS (0.2 nM (M_1R, M_2R) or 0.1 nM (M_4R)) and **79**, **80**-**82** or **85**-**88** at intact CHO-hM_xR cells (x = 1,2,4). In the case of **85**, **87** and **88**, competition binding experiments were also performed at CHO-hM_3R ([^{3}H]NMS: 0.2 nM) and CHO-hM_5R ([^{3}H]NMS: 0.3 nM) cells. Data represent mean values ± SEM from at least three independent experiments (each performed in triplicate).

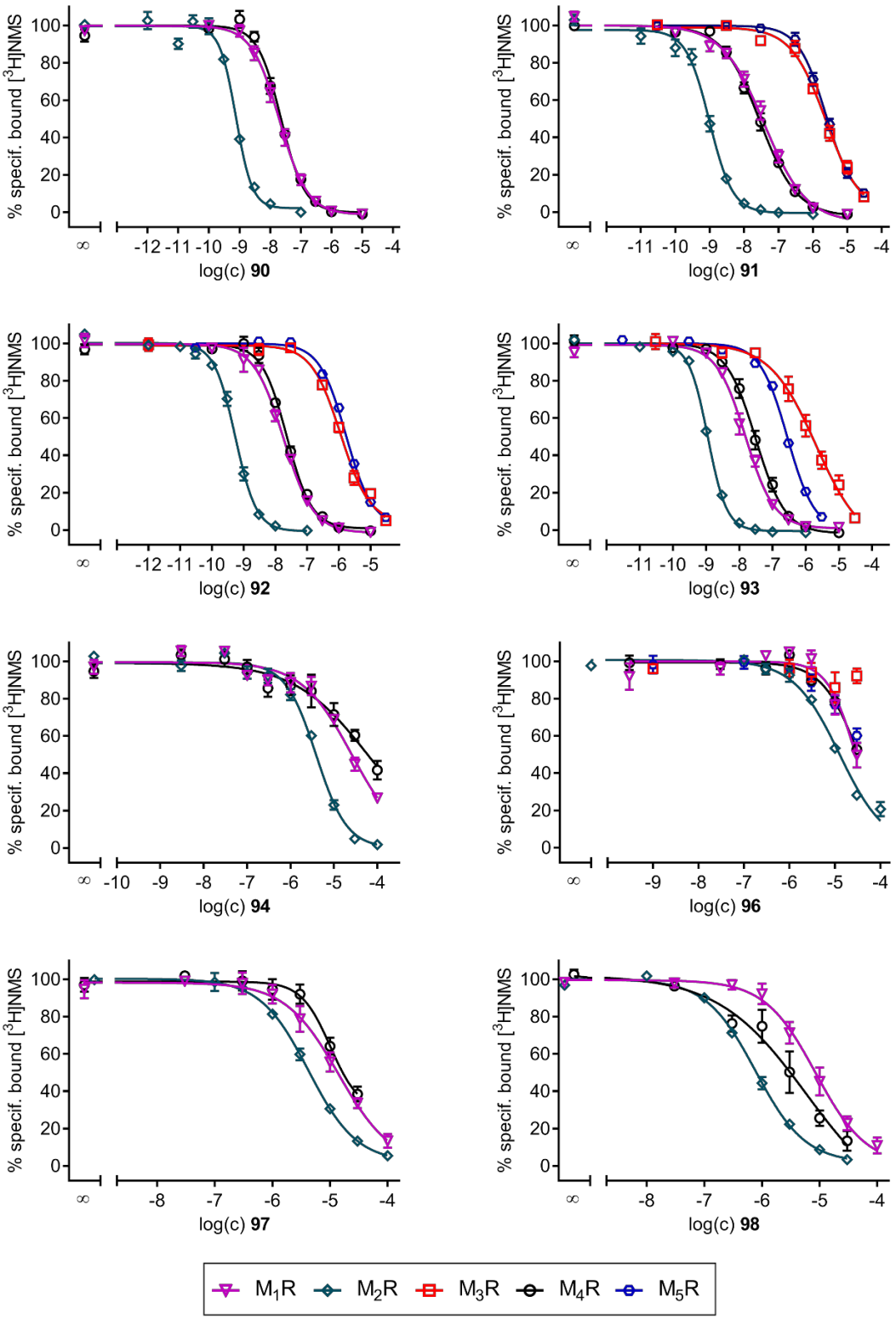


Figure S5. Radioligand displacement curves obtained from competition binding experiments with [^{3}H]NMS (0.2 nM (M_1R, M_2R) or 0.1 nM (M_4R)) and **90**-**94** or **96**-**98** at intact CHO-hM_xR cells (x = 1,2,4). In the case of **91**-**93** and **96** competition binding experiments were also performed at CHO-hM_3R ([^{3}H]NMS: 0.2 nM) and CHO-hM_5R ([^{3}H]NMS: 0.3 nM) cells. M_3R data of **96** could not be fitted. Data represent mean values ± SEM from at least three independent experiments (each performed in triplicate).

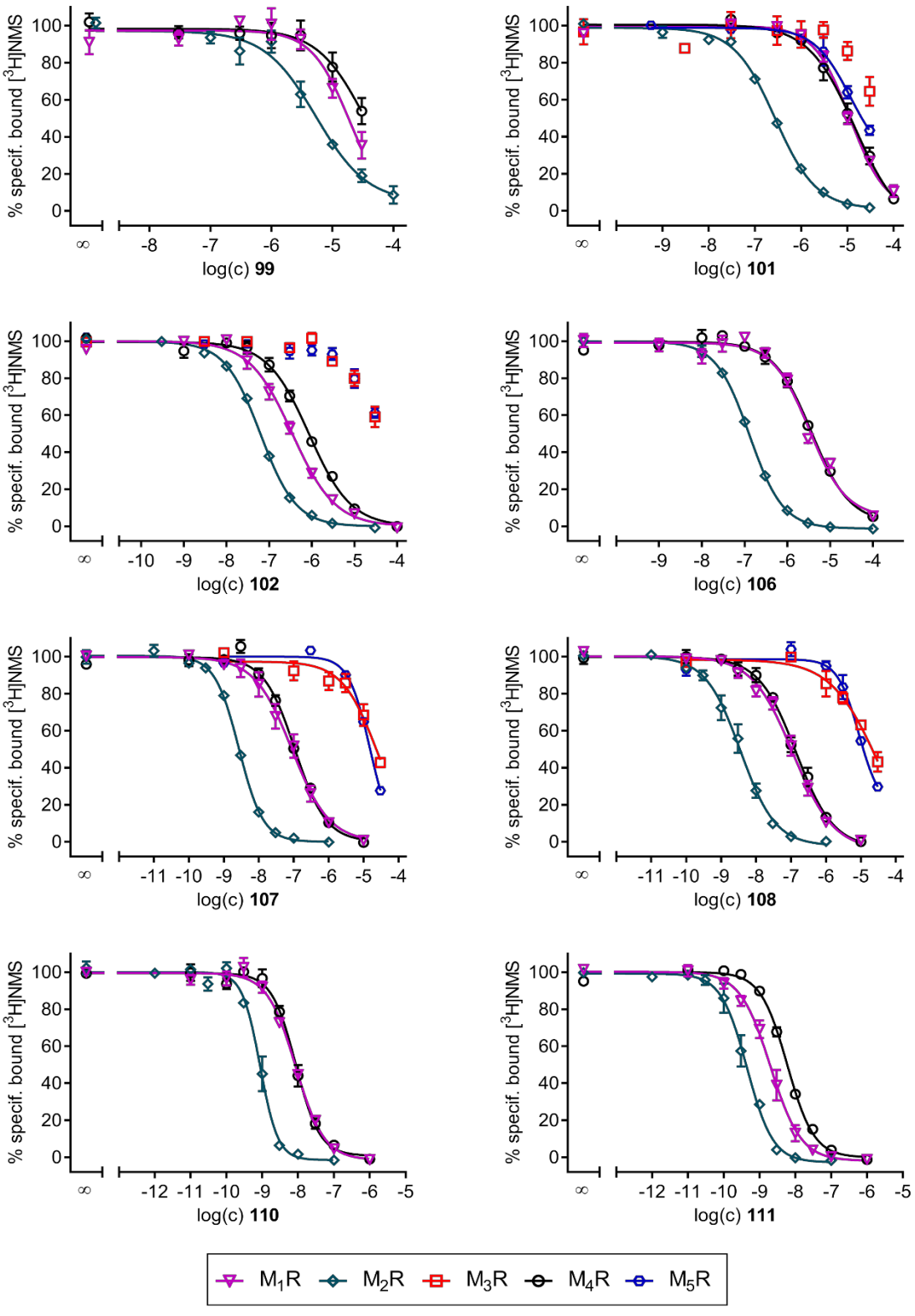


Figure S6. Radioligand displacement curves obtained from competition binding experiments with [^{3}H]NMS (0.2 nM (M_1R, M_2R) or 0.1 nM (M_4R)) and **99**, **101, 102, 106-108**, **110** or **111** at intact CHO-hM_xR cells (x = 1,2,4). In the case of **101**, **102**, **107** and **108,** competition binding experiments were also performed at CHO-hM_3R ([^{3}H]NMS: 0.2 nM) and CHO-hM_5R ([^{3}H]NMS: 0.3 nM) cells. M_3R data of **101** and **102** could not be fitted. M_5R data of **102** could not be fitted. Data represent mean values ± SEM from at least three independent experiments (each performed in triplicate).

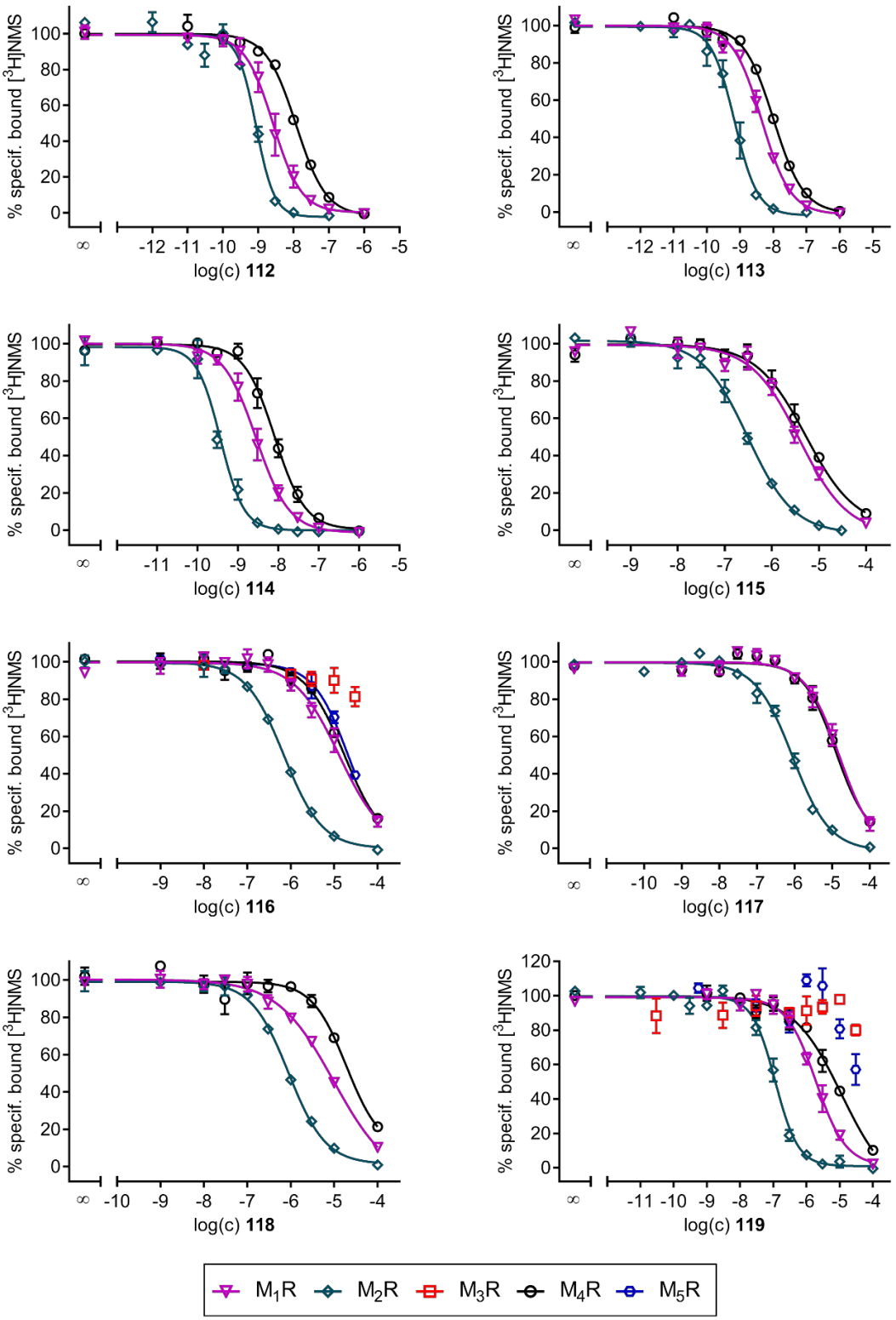


Figure S7. Radioligand displacement curves obtained from competition binding experiments with [^{3}H]NMS (0.2 nM (M_1R, M_2R) or 0.1 nM (M_4R)) and **112-119** at intact CHO-hM_xR cells (x = 1,2,4). In the case of **116** and **119**, competition binding experiments were also performed at CHO-hM_3R ([^{3}H]NMS: 0.2 nM) and CHO-hM_5R ([^{3}H]NMS: 0.3 nM) cells. M_3R data of **116** and **119** and M_5R data of **119** could not be fitted. Data represent mean values ± SEM from at least three independent experiments (each performed in triplicate).

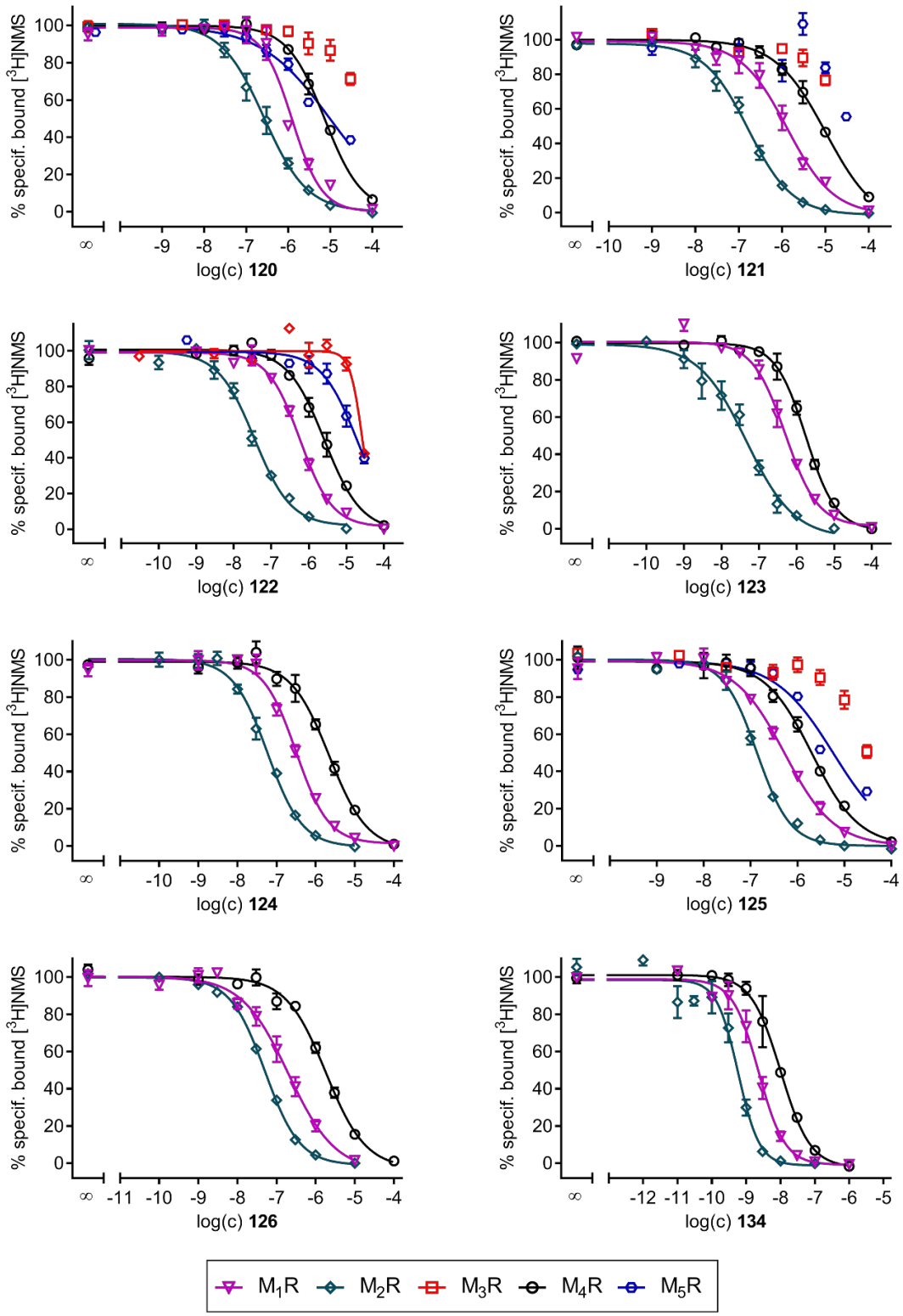


Figure S8. Radioligand displacement curves obtained from competition binding experiments with [^{3}H]NMS (0.2 nM (M_1R, M_2R) or 0.1 nM (M_4R)) and **120**-**126** or **134** at intact CHO-hM_xR cells (x = 1,2,4). In the case of **121**, **122** and **125**, competition binding experiments were also performed at CHO-hM_3R ([^{3}H]NMS: 0.2 nM) and CHO-hM_5R ([^{3}H]NMS: 0.3 nM) cells. M_3R and M_5R data of **121** and **125** and M_5R data of **121** could not be fitted. Data represent mean values ± SEM from at least three independent experiments (each performed in triplicate).

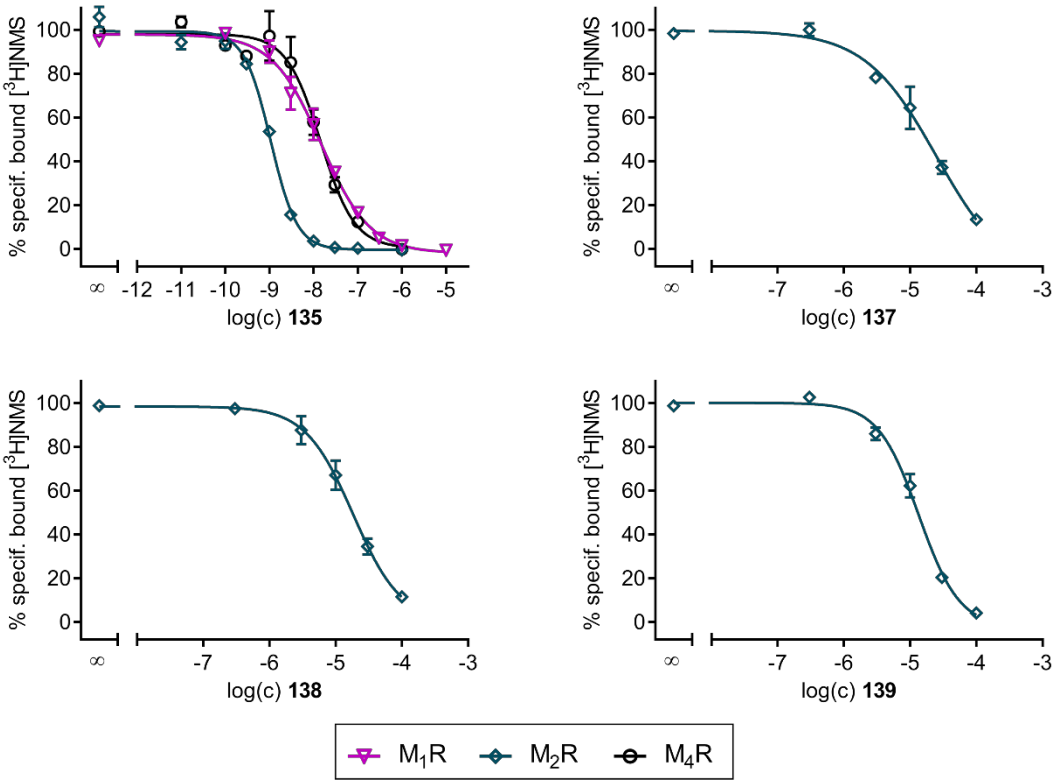


Figure S9. Radioligand displacement curves obtained from competition binding experiments with [^{3}H]NMS (0.2 nM (M_1R, M_2R) or 0.1 nM (M_4R)) and **135** and radioligand displacement curves obtained from competition binding experiments with [^{3}H]NMS (0.2 nM (M_1R, M_2R) and **137-139** at intact CHO-hM_2R cells. Data represent mean values ± SEM from at least three independent experiments (each performed in triplicate).

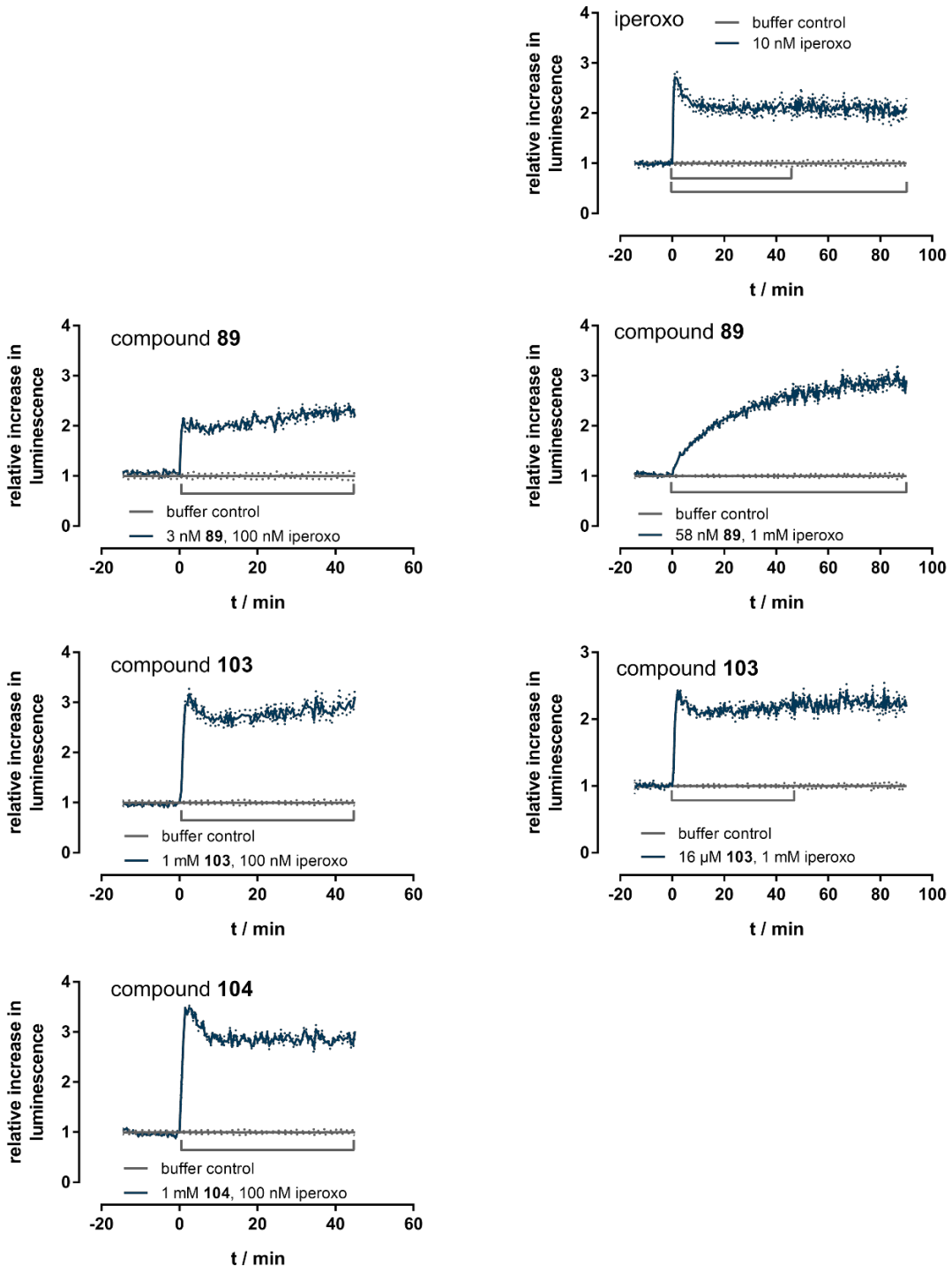
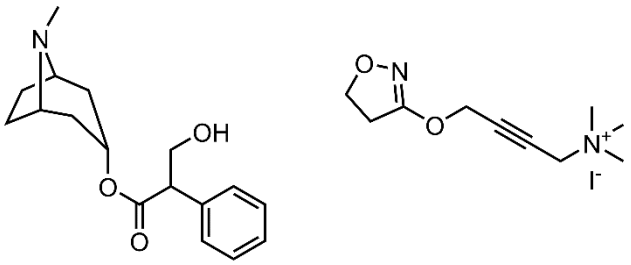


Figure S10. Representative time-courses of bioluminescence signals obtained from the M_2R mini-$G\alpha_i$-recruitment assay. Left column: time-courses obtained by stimulation with iperoxo (100 nM) in the presence of the M_2R antagonists **89**, **103** and **104** used at concentrations approximately corresponding to their IC_{50} values (obtained from the concentration-dependent inhibition of the effect elicited by 100 nM iperoxo, cf. Figure 2.4). Right column: time-courses obtained by stimulation with iperoxo alone or with iperoxo (1 mM) in the presence of antagonist **89** or **103** used at concentrations corresponding to 20× their IC_{50} (obtained from the concentration-dependent inhibition of the effect elicited by 100 nM iperoxo, *cf.* Figure 2.4). The grey bars indicate the signal window (45 or 90 min) which was used for analysis (calculation of the area under the curve).



atropine iperoxo

Figure S11. Structures of the MR antagonist atropine and the MR superagonist iperoxo.

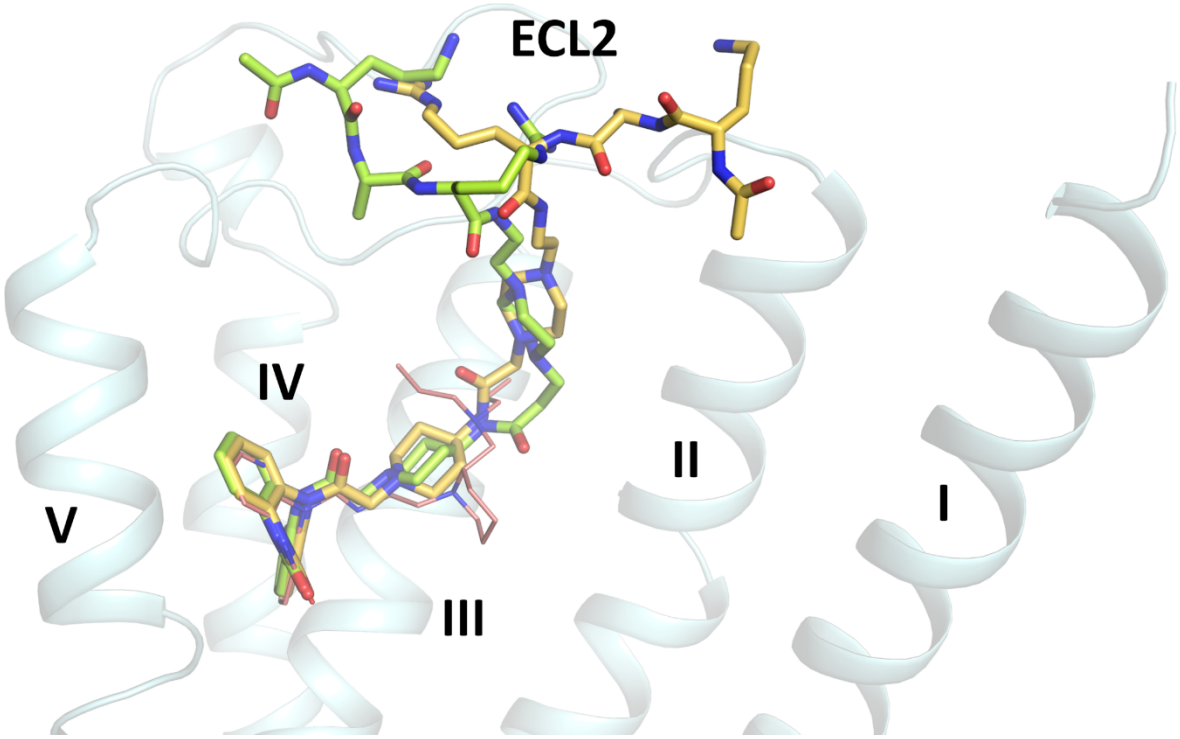


Figure S12. Overlay of the docking poses of **103** (orange) and **109** (green) in the M_2 inactive X-Ray structure (PDB-ID 5ZKB). The coordinates of AF-DX 384 (red, PDB-ID 5ZKB) are shown as reference.

M_1	---	E	R	T	V	L	A	G	Q	C	Y	I	Q	F	L	S	---
M_2	---	V	R	T	V	E	D	G	E	C	Y	I	Q	F	F	S	---
M_3	---	K	R	T	V	P	P	G	E	C	F	I	Q	F	L	S	---
M_4	---	K	R	T	V	P	D	N	Q	C	F	I	Q	F	L	S	---
M_5	---	K	R	T	V	P	L	D	E	C	Q	I	Q	F	L	S	---

Figure S13. Sequence alignment of the ECL2 for all human muscarinic receptors. The two glutamates interacting with **103** and **109** are highlighted in orange.

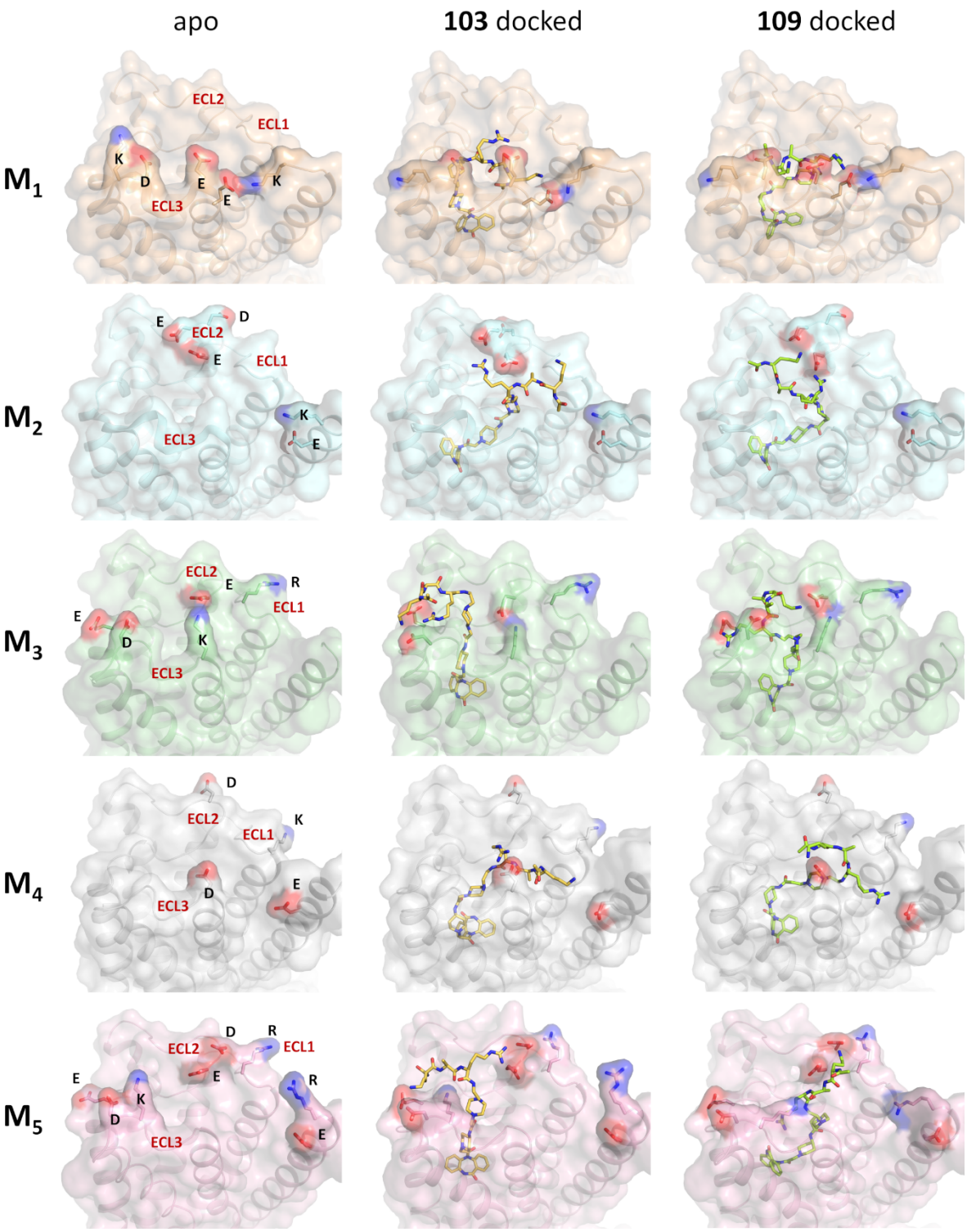


Figure S14. Surface presentation of the crystal structures of the M_1R (PDB-ID 5CXV), M_2R (PDB-ID 5ZKB), M_3R (PDB-ID 4U15), M_4R (PDB-ID 5DSG) and M_5R (PDB-ID 6OL9) without (apo) bound ligand and with bound **103** and **109** (induced-fit docking). In the extracellular region of the receptors, the anionic deprotonated carboxylic groups of glutamates (E) and aspartates (D) are shown in red and the cationic protonated amine/guanidine groups of lysines (K) and arginines (R) are shown in blue. In the case of all MR subtypes, the headgroup (piperidinylacetyl-dibenzodiazepinone moiety) of **103** and **109** was located in the orthosteric binding site, albeit adapting different orientations. In the case of the M_2R, the peptide moieties of **103** and **109** show interactions with two Glu residues in ECL2, which are absent in the M_1 and M_4 receptor (cf. 2.5. Supplementary Figure S13).

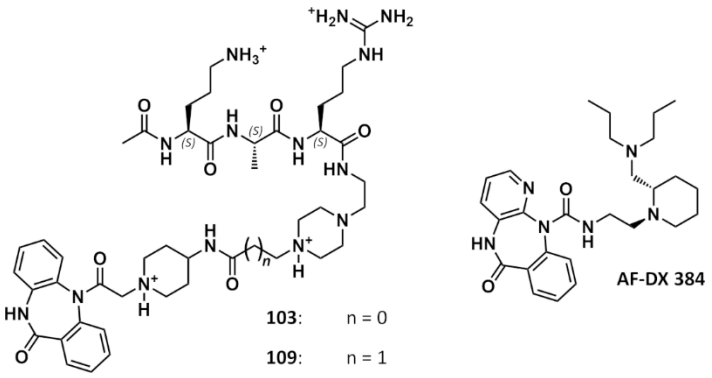


Figure S15. Chemical structures of **103**, **109** and AF-DX 384. **103** and **109** are shown in the protonation state defined for Induced-fit docking.

2. Analytical data of compound 6

Compound **6** was synthesized according to the reported procedure.[35] ^{1}H-NMR (600.3 MHz, DMSO-d_6): δ (ppm) 1.35-1.46 (m, 2H), 1.52-1.64 (m, 2H), 1.69-1.77 (m, 2H), 1.79-1.88 (m, 1H), 1.91-2.06 (m, 2H), 2.63 (d, *J* 6.8 Hz, 2H), 2.71-2.81 (m, 1H), 2.81-2.97 (m, 4H), 2.98-3.32 (m, 1H), 3.33-3.45 (m, 3H), 4.11 (brs, 2H), 6.58-6.63 (m, 1H), 6.72-6.77 (m, 1H), 6.99-7.03 (m, 1H), 7.12 (t, *J* 7.7 Hz, 1H), 7.44-7.50 (m, 2H), 7.66 (t, *J* 7.9 Hz, 1H), 7.76-7.79 (m, 1H), 7.91-7.94 (m, 1H), 7.95-7.97 (m, 2H), 8.01 (t, *J* 1.9 Hz, 1H), 9.13-9.37 (m, 1H) (note: one exchangeable proton could not be found). ^{13}C-NMR (150.9 MHz, DMSO-d_6): δ (ppm) 25.01, 25.98, 28.70, 30.76, 34.48, 35.77, 40.85, 42.20, 45.36, 48.66, 62.53, 116.03 (q, *J* 297 Hz, TFA), 116.03, 116.24, 119.74, 126.10, 126.81, 127.60, 127.74, 127.83, 130.15, 130.36, 130.51, 131.86, 133.71, 134.35, 138.16, 138.21, 143.26, 146.40, 158.23 (q, *J* 35 Hz, carbonyl group of TFA),162.30, 168.37. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{30}H_{35}ClN_3O_3S]^+$: 552.2082, found 552.2095. RP-HPLC (220 nm): 98% (t_R = 8.1 min, *k* = 9.7). $C_{30}H_{34}ClN_3O_3S \cdot C_4H_2F_6O_4$ (552.1300 + 228.04).

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CHAPTER 3

Further DIBA-peptide conjugates and stability studies in human plasma

The DIBA-peptide conjugates presented in this chapter were published together with DIBA-peptide conjugates prepared by Dr. Andrea Pegoli in the Journal of Medicinal Chemistry prior to the submission of this thesis:

A. Pegoli, D. Wifling, C.G. Gruber, X. She, H. Hubner, G. Bernhardt, P. Gmeiner, M. Keller, Conjugation of Short Peptides to Dibenzodiazepinone-Type Muscarinic Acetylcholine Receptor Ligands Determines M_2R Selectivity, *J. Med. Chem.*, 62 (**2019**) 5358-5369.

Author contributions:

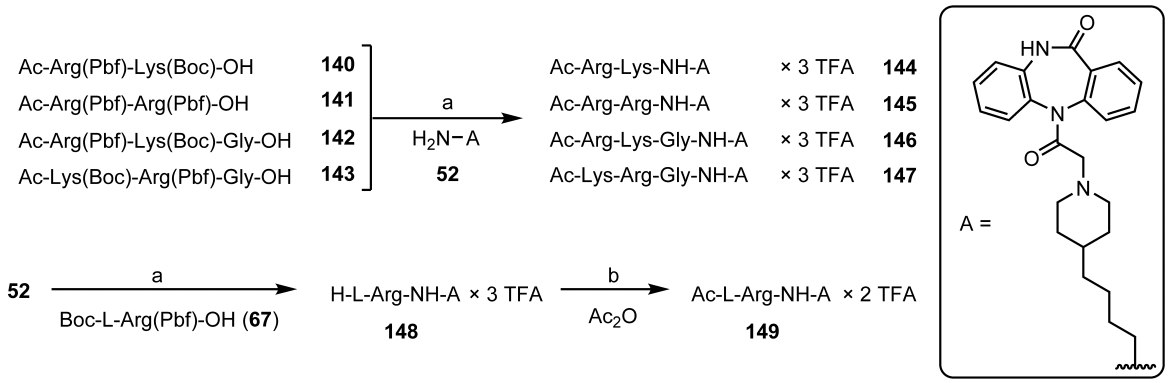
A.P., D.W., and C.G.G. contributed equally. A.P., C.G.G., M.K., and X.S. performed the syntheses and the analytical characterization of chemical compounds. A.P., C.G.G., and M.K. performed radioligand competition binding experiments and analyzed the data. M.K. initiated and planned the project. M.K. and G.B. supervised the research. M.K., A.P., D.W., G.B., and C.G.G. wrote the manuscript.

The rationale of the DIBA-peptide conjugation approach, which is aiming at highly selective dualsteric M_2R ligands, was described in Chapter 2. The series of DIBA-peptide conjugates described in Chapter 2 represents the continuation of the novel approach reported by Pegoli et al. in 2019.[1] In addition to the determination of M_1-M_5 receptor affinities in radioligand competition binding assays, one of the six presented dibenzodiazepinone derivatives as well as the DIBA-peptide conjugates UR-AP148 (**3**)[1] and UR-AP158 (**4**)[1] (for structure see Figure 2.1) were investigated with respect to stability in human plasma. Results and discussions

3.1. Results and Discussion

3.1.1. Chemistry

The N-terminally acetylated peptides **140**-**143** were synthesized on a chlorotrityl-resin (Fmoc strategy) according to the procedure procedure described in Chapter 2. Coupling of **140**-**143** to amine precursor **52** via the peptide C-termini, followed by cleavage of protecting groups, afforded the DIBA-peptide conjugates **144**-**147** (Scheme 3.1). Coupling of Boc-L-Arg(Pbf)-OH (**67**) to amine **52**, followed by deprotection, afforded **148**. Acetylation of **148** at the α-amino group to give **149** (Scheme 3.1).



Scheme 3.1. Synthesis of DIBA-peptide conjugates **144**-**147** and dibenzodiazepinone derivatives **148** and **149**. Reagents and conditions: (1) EDC, HOBt, DMF, 5 °C, 3 h, (2) TFA/H2O (95:5 v/v), rt, 3 h; (d) acetic anhydride, DIPEA, DMF, rt, 2 h, 78%; (e) DIPEA, DMF, rt, 30 min.

3.1.2. Radioligand competition binding studies

M_1-M_5 receptor affinities of **144**-**149** were determined by competition binding with the orthosteric MR antagonist [^{3}H]NMS using genetically engineered CHO cells (Table 3.1). Radioligand displacement curves are shown Figure 3.1. The M_2R selectivity of **144**-**149** over the M_1R and M_4R was less pronounced than towards the M_3R and M_5R. Compared to the structurally closely related DIBA-peptide conjugate **3**, the M_2R selectivity of **144**-**149** was generally lower (Table 3.1). This demonstrated that minor changes in the peptide moiety can have a marked effect on MR binding and M_2R selectivity, respectively.

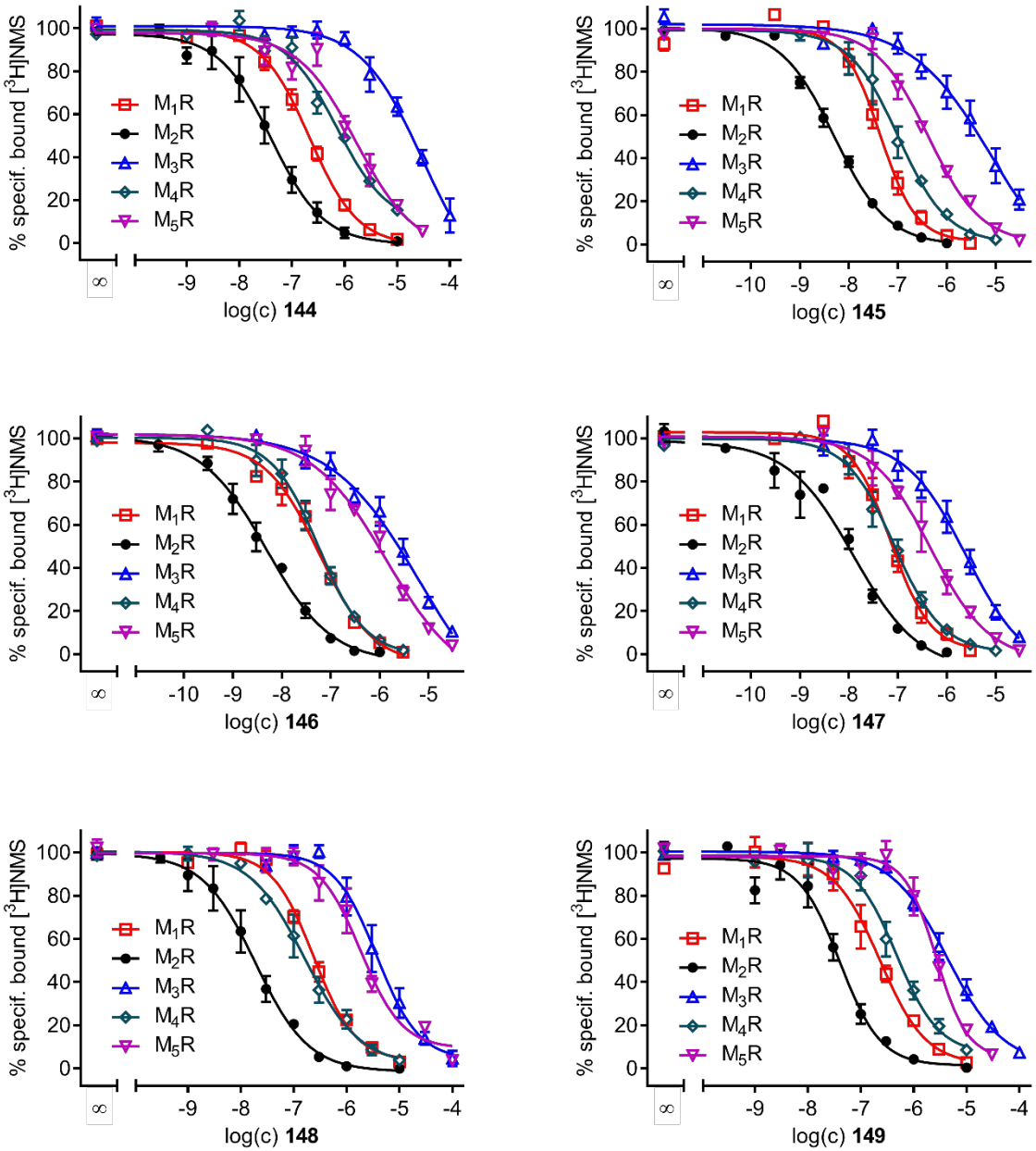


Figure 3.1. Displacement curves obtained from competition binding experiments with [^{3}H]NMS (0.2 nM (M_1R, M_2R, M_3R), 0.1 nM (M_4R) or 0.3 nM (M_5R)) and **144-149** at intact CHO-hM_xR cells (x = 1-5). Data represent mean values ± SEM from at least three independent experiments (each performed in triplicate).

Table 3.1. M_1-M_5 receptor binding data (pK_i values) of compounds **3** and **144-149**.[a]

cmpd.	structure (for A *cf.* Scheme 3.1)	pK_i					selectivity toward M_2R (ratios K_i ($M_{1,3,4,5}R$)/K_i (M_2R))			
		M_1R	M_2R	M_3R	M_4R	M_5R	M_1	M_3	M_4	M_5
3	Ac-Lys-Arg-NH-A	7.38 ± 0.14	8.97 ± 0.02	5.17 ± 0.07	7.21 ± 0.06	6.37 ± 0.07	49	6500	60	400
144	Ac-Arg-Lys-NH-A	7.02 ± 0.08	7.92 ± 0.18	5.26 ± 0.13	6.59 ± 0.06	6.30 ± 0.19	6.5	400	17	39
145	Ac-Arg-Arg-NH-A	7.70 ± 0.11	8.77 ± 0.03	6.02 ± 0.19	7.53 ± 0.16	6.79 ± 0.05	12	710	19	97
146	Ac-Arg-Lys-Gly-NH-A	7.64 ± 0.05	8.83 ± 0.10	6.11 ± 0.16	7.76 ± 0.03	6.44 ± 0.13	15	620	11	260
147	Ac-Lys-Arg-Gly-NH-A	7.49 ± 0.12	8.38 ± 0.05	6.19 ± 0.15	7.56 ± 0.10	6.70 ± 0.10	8.2	180	6.9	49
148	H-Arg-NH-A	6.97 ± 0.07	8.27 ± 0.12	5.90 ± 0.17	7.25 ± 0.11	5.62 ± 0.11	18	260	9.8	410
149	Ac-Arg-NH-A	7.01 ± 0.09	7.95 ± 0.09	5.83 ± 0.14	6.83 ± 0.10	6.01 ± 0.11	8.4	140	13	86

[a]Determined by competition binding with [^{3}H]NMS (K_d values/applied concentrations: M_1, 0.17/0.2 nM; M_2, 0.10/0.2 nM; M_3, 0.12/ 0.2 nM; M_4, 0.052/0.1 nM; M_5, 0.20/0.3 nM) at live CHO-hM_xR cells (x = 1-5) at 23 °C.

3.1.3. Stability studies in human plasma

Stabilities in human plasma/PBS (1:2 v/v) were studied for the DIBA-peptide conjugates **3**, **4** and **146**, which proved to be highly stable (> 99% intact compound after 24 h, Table 3.2). LC-MS analysis revealed that the Lys-Gly amide bond in **146** was the major cleavage site (structure of **146** see Scheme 3.1 or Table 3.1). Recoveries of the DIBA-peptide conjugates **3**, **4** and **146** from human plasma/PBS (1:2 v/v) were determined for two different concentrations (80 µM and 4 µM). In the case of the concentration of 80 µM, recoveries of **3**, **4** and **146** were in the range of 64-95%. The ratios of DIBA-peptide conjugate recovery over recovery of internal standard (**150**) are shown in Table 3.3 (see experimental section).

Table 3.2. Stabilities of **3**, **4** and **146** in human plasma/PBS (1:2 v/v) at 37 °C.[a]

	% intact peptide in plasma after the specified incubation times			
compd.	1 h	6 h	24 h	48 h
3	> 99	> 99	> 99	> 99
4	99 ± 3	> 99	> 99	> 99
146	> 99	> 99	> 99	83 ± 6

[a]The initial concentrations of compounds **3**, **4** and **146** in plasma/PBS (1:2 v/v) were 100 µM. Data represent the mean (± SEM) of three independent experiments (SEM not given when no decomposition was detected). LC-MS analysis revealed the Lys-Gly amide bond in **146** as the major cleavage site (structure of **146** *cf*. Scheme 3.1 or Table 3.1).

3.2. Experimental section

3.2.1. General experimental conditions

Acetonitrile (HPLC gradient grade) was obtained from Honeywell (Seelze, Germany) or Sigma-Aldrich (Taufkirchen, Germany). DMF, atropine, EDC, DIPEA, HFIP, 1-methyl-D-tryptophan (**150**) and TFA were purchased from Sigma-Aldrich. Absolute EtOH was obtained from Honeywell. CH_2Cl_2, HOBt, DMF for peptide synthesis and NMP for peptide synthesis were obtained from Acros Organics/Fisher Scientific (Nidderau, Germany). Boc-Arg(Pbf)-OH (**67**) was purchased from Carbolution Chemicals (St. Ingbert, Germany). Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, acetic anhydride and solid supports for SPPS (H-Arg(Pbf)-, H-Lys(Boc)-, and H-Gly-2-ClTrt PS resin) were purchased from Merck Chemicals (Darmstadt, Germany). Deuterated solvents were from Deutero (Kastellaun, Germany). HBTU and piperidine were from Iris Biotech (Marktredwitz, Germany). The synthesis of compound **52** was described elsewhere.[2] Millipore water was used for the preparation of buffers and HPLC eluents. 1.5- or 2-mL polypropylene reaction vessels with screw cap (Süd-Laborbedarf, Gauting, Germany) were used for small scale reactions, for the investigation of plasma stabilities and for the preparation and storage of stock solutions. Temperature-controlled reactions and plasma stabilities were performed in 1.5-mL reaction vessels using a Thermocell Mixing Block MB-102 from Bioer (Hangzhou, China). NMR spectra were recorded on a Bruker Avance III HD 600 equipped with a cryogenic probe (14.1 T, 1H: 600.1 MHz, 13C: 150.9 MHz) (Bruker, Karlsruhe, Germany). Abbreviations for the multiplicities of the signals are s (singlet), d (doublet), t (triplet), m (multiplet) and brs (broad-singlet). HRMS was performed on an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA USA) using an ESI source. Optical rotations at 589 nm (Na-D line) were measured on a. Preparative HPLC was performed with a system from Knauer (Berlin, Germany), composed of two K-1800 pumps and a K-2001 detector, or with a Prep 150 LC System from Waters (Eschborn, Germany) consisting of a

2545 Binary Gradient Module, a 2489 UV/Visible Detector and a Waters Fraction Collector III. When using acetonitrile as organic solvent, a Kinetex-XB C18 (5 µm, 250 mm × 21 mm; Phenomenex, Aschaffenburg, Germany) was used as stationary phase at a flow rate of 18 mL/min. Mixtures of 0.1% aq TFA and acetonitrile or mixtures of 0.1% aq TFA and MeOH were used as mobile phase. The detection wavelength was 220 nm. The solvent of collected fractions was removed by lyophilization. Analytical HPLC analysis was performed with a system from Agilent Technologies composed of a 1290 Infinity binary pump equipped with a degasser, a 1290 Infinity autosampler, a 1290 Infinity thermostated column compartment, a 1260 Infinity diode array detector, and a 1260 Infinity fluorescence detector. A Kinetex-XB C18 (2.6 µm, 100 × 3 mm; Phenomenex) was used as stationary phase at a flow rate of 0.6 mL/min. Mixtures of 0.04% aq TFA (A) and acetonitrile (B) were used as mobile phase. The following linear gradient was applied: 0-20 min: A/B 90:10-70:30, 20-22 min: 70:30-5:95, 22-26 min: 5:95.For all analytical HPLC runs the oven temperature was set to 25 °C, the injection volume was 20 µL and detection was performed at 220 nm. Compound concentrations were between 80 and 150 µM.

3.2.2. Compound characterization

Target compounds (**144**-**149**) were characterized by ^{1}H-NMR, ^{13}C-NMR and 2D-NMR (^{1}H-COSY, HSQC, HMBC) spectroscopy, HRMS and RP-HPLC analysis. The purity (HPLC, 220 nm) of all target compounds amounted to ≥97%).

Annotation concerning the ^{1}H- and ^{13}C-NMR spectra of **144**-**149**: due to a slow rotation about the exocyclic dibenzodiazepinone amide group on the NMR time scale, two isomers (ratios provided in the experimental protocols) were evident in the ^{1}H- and ^{13}C-NMR spectra.

Annotation concerning the ^{1}H-NMR spectra (solvent: DMSO-d_6) of **144**-**149**: in order to integrate signals interfering with the broad water signal at ca 3.5 ppm, spectra were additionally recorded in DMSO-d_6/D_2O (4:1 v/v) (spectra shown in Appendix).

3.2.3. Screening for pan-assay interference compounds (PAINS).

The search for PAINS in **144**-**149** via the public tool http://zinc15.docking.org/patterns/home[3] gave no hits.

3.2.4. Solid-phase peptide synthesis (SPPS)

SPPS was performed according to a previously described procedure.[1] Peptides **140**-**143** were used in the next step without purification. The chemical identities and purities of **140**-**143** were assessed by LC-HRMS (data not shown).

3.2.5. General procedure for the synthesis of compounds 144-147

The coupling of the side chain-protected peptides **140**-**143** to amine **52** was performed in 1.5- or 2-mL polypropylene reaction vessels with screw cap. HOBt (1.2 equiv.) and EDC (1.2 equiv.) were added to a cooled (5 °C) solution of the respective side chain-protected peptide (1 equiv.) in DMF (0.1-0.4 mL). A solution of **52** (free base, 1-1.2 equiv.) in DMF (0.1 mL) was immediately added and stirring was continued at 5 °C for 3 h. 1% aq TFA (0.1-0.4 mL, depending on the amount of DMF used to dissolve the peptide) was added and the mixture was subjected to preparative HPLC to isolate the protected intermediates (conditions not given). The latter were dissolved in TFA/H_2O (95:5 v/v). The mixtures were stirred at rt for 3 h, CH_2Cl_2 (ca 20 mL) was added and the volatiles were removed by evaporation. The final products were purified by preparative HPLC.

3.2.6. Experimental synthetic protocols and analytical data of compounds 144-149

S)-2-((*S*)-2-Acetamido-5-guanidinopentanamido)-6-amino-*N*-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b,e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)hexanamide tris(hydrotrifluoroacetate) (144)

Purification by preparative HPLC (gradient: 0-25 min: 0.1% aq TFA/acetonitrile 81:19-72:28, t_R = 8 min) yielded **144** as a white fluffy solid (16 mg, 0.015 mmol, 43%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) 1.11-1.19 (m, 2H), 1.20-1.39 (m, 8H), 1.40-1.54 (m, 7H), 1.58-1.68 (m, 2H), 1.69-1.82 (m, 2H), 1.86 (s, 3H), 2.70-2.78 (m, 2H), 2.85-2.95 (s, 1H), 2.96-3.15 (m, 5H), 3.21-3.29 (m, 1H), 3.52-3.60 (m, 1H), 3.76 (d, 0.6H, *J* 16 Hz), 3.90 (d, 0.4H, *J* 16 Hz), 4.11-4.23 (m, 2H), 4.33-4.49 (m, 1H), 7.03 (brs, 2H), 7.21-7.31 (m, 2H), 7.31-7.64 (m, 5H), 7.64-7.94 (m, 9H), 8.05-8.10 (m, 1H), 9.56 (brs, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H).^{13}C-NMR (150.9 MHz, MeOH-d_4): δ (ppm) 22.24, 22.47, 23.21, 25.02, 26.55, 28.72, 28.86, 29.06, 31.40, 32.74, 34.93, 38.41, 38.64, 40.39, 52.27, 52.77, 53.22, 55.93, 121.87, 122.29, 124.87, 125.46, 127.25, 127.70, 128.25, 128.69, 128.95, 129.98, 130.35, 130.92, 131.51, 131.61, 133.02, 133.75, 134.66, 135.74, 140.98, 142.70, 156.77, 165.73, 166.03, 169.70, 171.10, 171.40 (note: ^{13}C-signal of the exocyclic carbonyl group, attached to the diazepinone ring, not apparent). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{38}H_{57}N_{10}O_5]^+$

733.4508, found 733.4504. RP-HPLC (220 nm): 97% (t_R = 6.4 min, k =7.4). $C_{38}H_{56}N_{10}O_5 \cdot C_6H_3F_9O_6$ (732.93 + 342.07).

(*S*)-2-Acetamido-5-guanidino-*N*-((*S*)-5-guanidino-1-oxo-1-((4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b,e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)amino)pentan-2-yl)pentanamide tris(hydrotrifluoroacetate) (145)

Purification by preparative HPLC (gradient: 0-25 min: 0.1% aq TFA/acetonitrile 81:19-72:28, t_R = 8 min) yielded **145** as a white fluffy solid (20 mg, 0.018 mmol, 52%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) 1.14-1.26 (m, 4H), 1.28-1.57 (m, 11H), 1.57-1.69 (m, 2H), 1.70-1.83 (m, 2H), 1.85 (s, 3H), 2.80-2.95 (m, 1H), 2.95-3.02 (m, 2H), 3.02 -3.14 (m, 5H), 3.29-3.37 (m, 1H), 3.57-3.64 (m, 1H), 3.76 (d, 0.6H, *J* 16 Hz), 3.90 (d, 0.4H, *J* 16 Hz), 4.14-4.25 (m, 2H), 4.35-4.47 (m, 1H), 7.01 (brs, 4H), 7.21-7.41 (m, 5H), 7.41-7.55 (m, 3H), 7.55-7.62 (m, 0.6H), 7.62-7.80 (m, 4.4H), 7.81-7.83 (m, 0.6H), 7.83-7.87 (m, 1H), 7.87-7.90 (m, 0.4H), 7.92-7.96 (m, 1H), 8.05-8.10 (m, 1H), 9.55 (brs, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, MeOH-d_4): δ (ppm) 22.24, 23.22, 25.03, 25.07, 28.70, 28.87, 29.06, 29.22, 32.74, 34.92, 38.42, 40.31, 40.38, 52.10, 52.35, 52.78, 53.23, 55.93, 121.87, 122.29, 124.89, 125.47, 127.24, 127.70, 128.23, 128.71, 128.97, 129.56, 129.68, 130.00, 130.35, 130.85, 130.93, 131.55, 133.04, 133.76, 134.66, 135.75, 140.96, 156.75 (2 × guanidinium group), 157.8 (broad signal, carbonyl group of TFA), 165.73, 166.03, 169.68, 170.87, 171.45. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{38}H_{57}N_{12}O_5]^+$ 761.4569, found 761.4556. RP-HPLC (220 nm): 97% (t_R = 6.8 min, k =7.9). $C_{38}H_{56}N_{12}O_5 \cdot C_6H_3F_9O_6$ (760.95 + 342.07).

(*S*)-2-((*S*)-2-Acetamido-5-guanidinopentanamido)-6-amino-*N*-(2-oxo-2-((4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b,e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)amino)ethyl)hexanamide tris(hydrotrifluoroacetate) (146)

Purification by preparative HPLC (gradient: 0-25 min: 0.1% aq TFA/acetonitrile 81:19-72:28, t_R = 8 min) yielded **146** as a white fluffy solid (16 mg, 0.014 mmol, 35%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) 1.12-1.19 (m, 2H), 1.19-1.26 (m, 2H), 1.26-1.40 (m, 6H), 1.40-1.58 (m, 7H), 1.58-1.71 (m, 2H), 1.70-1.76 (m, 1H), 1.76-1.83 (m, 1H), 1.85 (s, 3H), 2.71-2.79 (m, 2H), 2.85-2.97 (m, 1H), 2.97-3.13 (m, 5H), 3.29-3.35 (m, 1H), 3.58-3.70 (m, 3H), 3.76 (d, 0.6H, *J* 16 Hz), 3.90 (d, 0.4H, *J* 16 Hz), 4.15-4.27 (m, 2H), 4.33-4.48 (m, 1H), 7.06 (brs, 2H), 7.21-7.57 (m, 6.6H), 7.57-7.62 (m, 0.4H), 7.63-7.80 (m, 7H), 7.80-7.84 (m, 0.6H), 7.86-7.91 (m, 0.4H), 8.02-8.09 (m, 2H), 8.09-8.14 (m, 1H), 9.56 (brs, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H).

^{13}C-NMR (150.9 MHz, MeOH-d_4): δ (ppm) 22.08, 22.42, 23.24, 24.96, 26.52, 28.67, 28.85, 29.10, 31.00, 32.74, 34.92, 38.43, 38.62, 40.39, 41.85, 52.20, 52.44, 52.76, 53.21, 55.89, 121.85, 122.27, 124.86, 125.44, 126.72, 127.21, 127.68, 128.23, 128.67, 128.95, 129.66, 129.98, 130.33, 130.90, 130.93, 131.51, 133.00, 133.73, 134.64, 135.73, 140.96, 156.76, 166.00, 168.31, 169.59, 171.60, 171.70 (note: ^{13}C-signal of the exocyclic carbonyl group, attached to the diazepinone ring, not apparent). HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{40}H_{60}N_{11}O_6]^+$ 790.4723, found 790.4730. RP-HPLC (220 nm): 98% (t_R = 6.4 min, *k* =7.4). $C_{40}H_{59}N_{11}O_6 \cdot C_6H_3F_9O_6$ (789.98 + 342.07).

(*S*)-2-Acetamido-6-amino-*N*-((*S*)-5-guanidino-1-oxo-1-((2-oxo-2-((4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b,e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)amino)ethyl)amino)pentan-2-yl)hexanamidetris (hydrotrifluoroacetate) (147)

Purification by preparative HPLC (gradient: 0-25 min: 0.1% aq TFA/acetonitrile 81:19-72:28, t_R = 8 min) yielded **147** as a white fluffy solid (21 mg, 0.019 mmol, 54%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ^{1}H-NMR (600 MHz, DMSO-d_6): δ (ppm) 1.12-1.19 (m, 2H), 1.19-1.40 (m, 8H), 1.40-1.58 (m, 7H), 1.58-1.66 (m, 1H), 1.66-1-77 (m, 2H), 1.77-1.83 (m, 1H), 1.85 (s, 3H), 2.71-2.79 (m, 2H), 2.85-2.96 (m, 1H), 2.97-3.13 (m, 5H), 3.30-3.36 (m, 1H), 3.58-3.70 (m, 3.6H), 3.76 (d, 0.4H, *J* 17 Hz), 4.17-4.25 (m, 2H), 4.35-4.47 (m, 1H), 7.01 (brs, 2H), 7.19-7.57 (m, 6.6H), 7.57-7.62 (m, 0.4H), 7.62-7.80 (m, 7H), 7.80-7.85 (m, 0.6H), 7.86-7.91 (m, 0.4H), 8.02-8.10 (m, 2H), 8.10-8.15 (m, 1H), 9.57 (brs, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, MeOH-d_4): δ (ppm) 22.35, 22.44, 23.26, 24.92, 26.65, 28.67, 28.80, 29.12, 31.09, 32.75, 34.94, 38.46, 38.70, 40.35, 41.88, 52.26, 52.44, 52.63, 52.77, 53.23, 55.93, 121.87, 122.30, 124.88, 125.46, 126.74, 127.26, 127.34, 127.71, 128.25, 128.71, 129.03, 129.66, 129.68, 130.00, 130.35, 130.93, 131.60, 133.03, 133.76, 134.66, 135.75, 139.42, 140.96, 156.77, 157.5 (broad signal, carbonyl group of TFA), 163.68, 164.18, 165.74, 166.02, 168.26, 169.57, 171.50, 172.03. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{40}H_{60}N_{11}O_6]^+$ 790.4723, found 790.4730. RP-HPLC (220 nm): 97% (t_R = 6.5 min, *k* =7.5). $C_{40}H_{59}N_{11}O_6 \cdot C_6H_3F_9O_6$ (789.98 + 342.07).

(S)-2-Amino-5-guanidino-N-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)pentanamide tris(hydrotrifluoroacetate) (148)

Compound **148** was prepared from amine **52** (55.6 mg, 0.137 mmol) and compound **67** (60 mg, 0.114 mmol) according to the general procedure for the synthesis of **144**-**147**. Purification by preparative HPLC (gradient: 0-25 min: 0.1% aq TFA/acetonitrile 81:19-72:28, t_R = 7 min) yielded **148** as a white fluffy solid (67 mg, 0.074 mmol, 65%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. 1H-NMR (600 MHz, DMSO-d6): δ (ppm) 1.13-1.29 (m, 4H), 1.30-1.52 (m, 7H), 1.64-1.71 (m, 2H), 1.71-1.83 (m, 2H), 2.83-2.96 (m, 1H), 2.96-3.04 (m, 1H), 3.04-3.14 (m, 4H), 3.28-3.35 (m, 0.8H), 3.55-3-63 (m, 1.2H), 3.65-3.73 (m, 1H), 3.75 (d, 0.6H, *J* 17 Hz), 3.90 (d, 0.4H, *J* 17 Hz), 4.32-4.48 (m, 1H), 6.95-7.49 (m, 7H), 7.49-7.56 (m, 1.4H), 7.56-7.63 (m, 0.6H), 7.63-7.79 (m, 2H), 7.79-7.92 (m, 2H), 8.11-8.24 (m, 3H), 8.41-8.48 (m, 1H), 9.57 (brs, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H). ^{13}C-NMR (150.9 MHz, MeOH-d_4): δ (ppm) 22.32, 24.20, 28.31, 28.74, 28.98, 32.81, 34.95, 38.67, 40.08, 51.94, 52.80, 53.24, 55.96, 116.05 (TFA), 118.04 (TFA), 121.91, 122.34, 124.93, 125.51, 126.75, 127.27, 127.71, 128.75, 129.00, 129.71, 130.04, 130.38, 130.97, 131.63, 133.07, 133.80, 134.68, 135.77, 140.99, 156.84, 158.3 (q, *J* 32 Hz, carbonyl group of TFA), 163.70, 164.35, 165.78, 166.07, 168.05, 171.50. HRMS (ESI): m/z $[M+H]^+$ calcd. for $[C_{30}H_{43}N_8O_3]^+$ 563.3453, found 563.3462. RP-HPLC (220 nm): 99% (t_R = 6.2 min, k =7.1). $C_{30}H_{42}N_8O_3 \cdot C_6H_3F_9O_6$ (562.72 + 342.07).

(S)-2-Acetamido-5-guanidino-N-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)pentanamide bis(hydrotrifluoroacetate) (149)

Acetyl anhydride (3.6 µL, 0.038 mmol) was added to a solution of **149** (25 mg, 0.032 mmol) and DIPEA (28 µL, 0.16 mmol) in DMF (0.3 mL) and the mixture was stirred at rt for 2 h. Isolation of the product by preparative HPLC (gradient: 0-25 min: 0.1% aq TFA/acetonitrile 81:19-72:28, t_R = 9 min) afforded **149** as a white fluffy solid (20.8 mg, 0.025 mmol, 78%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. 1H-NMR (600 MHz, DMSO-d6): δ (ppm) 1.13-1.26 (m, 4H), 1.30-1.50 (m, 8H), 1.57-1.66 (m, 1H), 1.69-1.82 (m, 2H), 1.83 (s, 3H), 2.85-2.96 (m, 1H), 2.96-3.14 (m, 5H), 3.28-3.35 (m, 1H), 3.57-3.61 (m, 1H), 3.76 (d, 0.6H, *J* 16 Hz), 3.91 (d, 0.4H, *J* 16 Hz), 4.13-4.21 (m, 1H), 4.33-4.48 (m, 1H), 7.03 (brs, 2H), 7.20-7.31 (m, 2H), 7.31-7.39 (m, 1.6H), 7.39-7.49 (m, 1.4H), 7.49-7.55 (m, 1.4H), 7.55-7.62 (m, 0.6H), 7.62-7.79 (m, 3H), 7.79-7.85 (m, 0.6H), 7.85-7.93 (m, 1.4H), 7.95-8.02 (m, 1H), 9.55 (brs, 1H), 10.73 (s, 0.4H), 10.78 (s, 0.6H). 13C-NMR (150.9 MHz, MeOH-d_4): δ (ppm) 22.49, 25.16, 28.69, 29.09, 29.26, 32.74, 34.90, 38.31, 40.33, 52.13,

52.65, 52.78, 53.23, 55.93, 116.05 (TFA), 118.10 (TFA), 121.88, 122.30, 124.89, 125.47, 126.74, 127.25, 127.70, 128.25, 128.70, 128.95, 129.69, 130.00, 130.36, 131.61, 133.03, 133.76, 134.68, 135.77, 140.97, 156.77, 158.2 (broad signal, carbonyl group of TFA), 163.67, 164.17, 165.73, 166.03, 169.19, 171.22. HRMS (ESI): m/z $[M+H]^+$ calcd. for $[C_{32}H_{45}N_8O_4]^+$ 605.3558, found 605.3560. RP-HPLC (220 nm): 98% (t_R = 8.0 min, k = 9.5). $C_{32}H_{44}N_8O_4 \cdot C_4H_2F_6O_4$ (604.76 + 228.05).

3.2.7. Cell culture

CHO-K9 cells, stably transfected with the DNA of human muscarinic receptors M_1-M_5 (obtained from Missouri S&T cDNA Resource Center; Rolla, MO) were cultured in HAM's F12 medium supplemented with fetal calf serum (Biochrom, Berlin, Germany) (10%) and G418 (Biochrom) (750 µg/mL).

3.2.8. Radioligand competition binding

Competition binding experiments were performed as described in Chapter 2.

3.2.9. Investigation of the stability of 3, 4 and 146 in human plasma.

The metabolic stabilities of **3**, **4** and **146** were investigated in human blood plasma/PBS pH 7.4 (1:2 v/v) (in the following referred to as plasma/PBS) at 37 °C. 1-Methyl-D-tryptophan (**150**) was used as internal standard. As the purity (RP-HPLC, 220 nm) of **150** was < 95% (data not shown) it was purified by preparative HPLC to give a purity of > 99%. Plasma was obtained by the collection of human blood from a healthy donor in 5.5-mL heparinized plasma-monovettes, followed by centrifugation at 1200 g at 4 °C for 10 min. The supernatants were pooled in two 50-mL Falcon tubes and centrifuged again at 1200 g at 4 °C for 10 min. The plasma was aliquoted and stored at −80 °C.

For the addition of the compounds to plasma/PBS, 5 mM stock solutions in acetonitrile/0.04% aqueous TFA (3:7 v/v) were used. Recoveries were determined for concentrations of 80 µM and 4 µM, and an internal standard concentration of 10 µM. For this purpose, **150** and the DIBA-peptide conjugates were added to plasma/PBS (total volume: 70 µL), immediately followed by vortexing (ca 10 s) and precipitation of protein by the addition of EtOH/acetonitrile (1:1 v/v) (140 µL). The mixture was vortexed for 5 min und centrifuged at 16,100 g at 4 °C for 10 min. Aliquots (180 µL) of the supernatant were transferred into 1.5-mL polypropylene reaction vessels containing 10% aqueous TFA (5 µL). The volatiles were removed in a vacuum concentrator under reduced pressure at 40 °C (ca

60 min), and the residue was taken up in acetonitrile/0.04% aqueous TFA (1:9 v/v) (90 µL) by vortexing (2 min). The samples were filtrated using a 0.2-µm RC-membrane filter (Phenomenex, Aschaffenburg, Germany) and analyzed by RP-HPLC using the analytical HPLC system and the conditions as described under general experimental conditions, but applying the following gradient: 0–12 min: A/B 90:10–70:30, 12–16 min: 70:30–5:95, 16–20 min: 5:95. On the day of an experiment four-point calibrations were performed for the respective DIBA-peptide conjugates and the internal standard. Peak areas representing 100% recovery were obtained by analyzing 53.3 µM and 2.67 µM DIBA-peptide solutions as well as a 6.67 µM solution of **150** (in duplicate each). All peak areas were transformed into concentrations (µM) and percent recoveries of **3**, **4**, **146** and **150** were calculated based on the average values of the 100% reference samples (see Table 3.3). Recovery ratios were obtained by dividing the recovery of the DIBA-peptide conjugate by the recovery of **150** for each individual sample (n = 3 or 4, *cf.* Table 3.3).

For the investigation of the stabilities in plasma, the DIBA-peptide conjugates (**3**, **4**, **146**) and **150** were added to plasma/PBS at a concentration of 100 µM and 10 µM, respectively (in triplicate each). After 1, 6, 24 and 48 h of incubation under shaking at 37 °C, aliquots (70 µL) were taken and processed as described above for the determination of recoveries. On the day of an experiment four-point calibrations were performed for the respective DIBA-peptide conjugates and **150**. Peak areas representing 100% recovery were obtained by analyzing 66.7 µM 'DIBA-peptide' solutions and a 6.67 µM solution of **150** (in duplicate). Based on the calibration, the peak areas of the 100% references and of the samples were transformed into concentrations (µM). Recoveries of **3**, **4** and **146** were calculated by multiplying the recovery of **150**, obtained for each individual sample, with the recovery ratio obtained for the concentration of 80 µM (see above). The plasma concentrations of the DIBA-peptide conjugates were obtained by dividing the determined 'DIBA-peptide' concentration by the respective recovery. Degradation (%) of **3**, **4** and **146** was calculated based on the average values of the 100% reference samples.

Note: recovery ratios obtained for a concentration of 4 µM were not used as **3**, **4** and **146** exhibited high stabilities (lowest concentration in plasma ca 80 µM (**146**, 48 h)).

Table 3.3. Recoveries of DIBA-peptide conjugates **3**, **4** and **146** from human plasma/PBS (1:2 v/v) for two different concentrations (80 µM and 4 µM), and ratios of 'DIBA-peptide' recovery over recovery of internal standard (**150**).

compd.	'DIBA-peptide' concentration 80 µM			'DIBA-peptide' concentration 4 µMa		
	recovery 'DIBA-peptide' (%)[a]	recovery I.S. (**150**) (%)[a]	ratio[b]	recovery 'DIBA-peptide' (%)[a]	recovery I.S. (**150**) (%)[a]	ratio[b]
3	75	101	0.75	60	98	0.62
	91	101	0.90	59	85	0.69
	86	102	0.85	58	88	0.66
			(0.83 ± 0.05)	56	95	0.59
						(0.64 ± 0.02)
4	64	96	0.67	87	98	0.89
	95	100	0.95	86	98	0.89
	79	98	0.81	86	91	0.94
	81	99	0.82	90	103	0.88
			(0.81 ± 0.06)			(0.90 ± 0.02)
146	75	87	0.87	41	83	0.49
	76	89	0.86	40	81	0.50
	72	95	0.75	44	73	0.61
	72	92	0.78			(0.53 ± 0.04)
			(0.81 ± 0.03)			

[a]Recoveries of the DIBA-peptide conjugates and of the internal standard (I.S.) from human plasma/PBS (1:2 v/v) using a DIBA-peptide concentration of 80 µM or 4 µM and an I.S. concentration of 10 µM (five independent experiments). [b]Ratios of DIBA-peptide recovery over recovery of I.S. calculated for individual experiments, as well as mean recovery ratios ± SEM (given in parenthesis).

3.2.10. Data processing

Retention (capacity) factors were calculated from retention times (t_R) according to $k = (t_R - t_0)/t_0$ (t_0 = dead time). Data from radioligand ([^{3}H]NMS) competition binding assays were processed as reported previously.[4] K_i values for the calculation of relative MR affinities (M_2R selectivity, Table 3.1) were obtained by transforming pIC_{50} values from individual competition binding experiments with [^{3}H]NMS to IC_{50} values followed by conversion to K_i values according to the Cheng-Prusoff equation[5] and calculation of mean K_i values.

3.3. References

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CHAPTER 4

Fluorescently labeled dibenzodiazepionone-type muscarinic M_2R ligands

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Corinna G. Gruber, Andrea Pegoli, Christoph Müller, Lukas Grätz, Xueke She, Max Keller. Differently fluorescence-labelled dibenzodiazepinone-type muscarinic acetylcholine receptor ligands with high M_2R affinity. *RSC Med. Chem*., 11 (**2020**), 823-832. Reproduced with permission from RSC Medicinal Chemistry.

Author contributions:

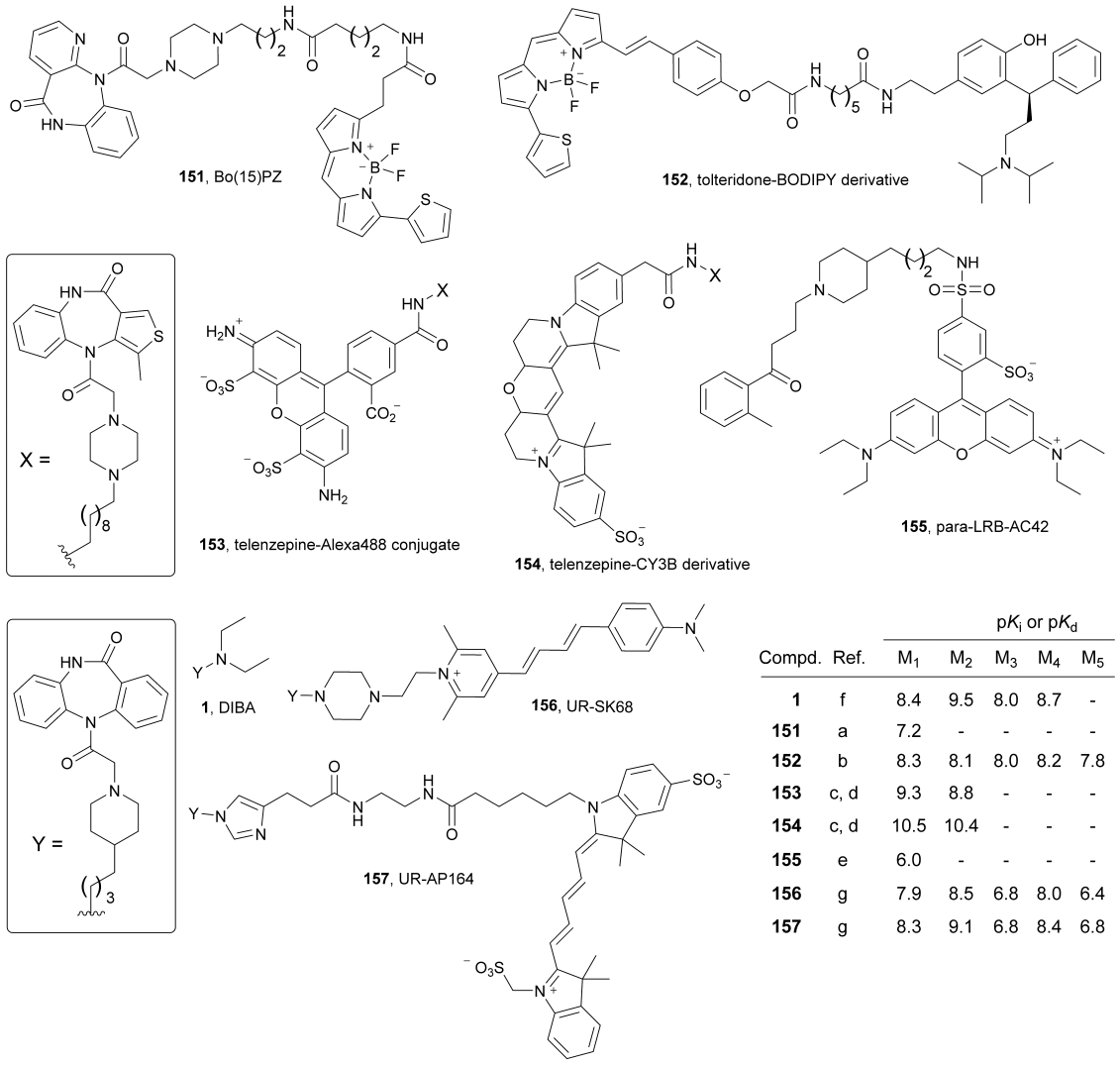
C.G.G., A.P. and X.S. synthesized the fluorescent ligands. C.G.G. performed radiochemical and flow cytometric binding experiments and analysed the data. M.K. initiated the project and supervised the research. C.G.G. and M.K. wrote the manuscript. All authors have given approval to the final version of the manuscript.

4.1. Introduction

In the past decades, fluorescence-based techniques have been increasingly used to study membrane receptors such as G-protein coupled receptors (GPCRs), including the analysis of ligand-receptor interactions as well as the investigation of receptor expression, structure and function.[1-4] Besides fluorescently tagged receptors, fluorescent ligands represent interesting molecular tools, which can also be used to study endogenously expressed receptors.[1] Therefore, there is a growing demand of suitable fluorescent GPCR ligands. Compared with radioligands, fluorescent ligands exhibit several advantages, such as less problems concerning safety risks, legal issues and waste disposal. Furthermore, they are applicable to techniques, which have become routine in many laboratories, for instance fluorescence microscopy and flow cytometry. Typically, fluorescent ligands are composed of a pharmacophore, mediating receptor affinity, a spacer/linker moiety and the fluorophore.[1, 5] The design of fluorescent ligands is not trivial, e.g. with respect to high receptor affinity and favorable physicochemical properties. Crucial factors are the type of the fluorescent dye (size, lipophilicity, spectroscopic and bleaching properties, etc.) and the point of attachment, structure and length of the linker.[1, 6-11]

Numerous fluorescent probes have been reported for GPCRs, for instance for histamine,[12-16] dopamine,[17, 18] opioid,[19-22] neuropeptide Y,[23-28] adenosine,[29] and neurotensin[30, 31] receptors. Concerning muscarinic acetylcholine receptors (MRs), various fluorescent MR ligands were reported, e.g. the BODIPY558/568-labelled derivative **151**,[32] derived from the M_1 subtype preferring MR antagonist pirenzepine, the BODIPY630/650-labelled tolterodine derivative **152**,[33] the Alexa488-labelled telenzepine derived MR antagonist **153**,[34, 35] the Cy3B-labelled telenzepine analog **154**,[34-36] and the lissamine rhodamine B-labelled AC-42 derivative **155**[37] (Figure 4.1; note: in the case of **151** and **153**-**155** further congeners, containing the same pharmacophore but different fluorophores such as Cy3, Cy5 Cascade Blue or 6-carboxyfluorescein and, in part, also different linkers, were reported[32, 36-41]). The recent finding that replacement of the diethylamine moiety in the M_2R preferring dibenzodiazepinone-type MR antagonist DIBA[42] (**1**, Figure 4.1) by bulky moieties was well tolerated with respect to M_2R binding,[43-46] gave rise to prepare a series of DIBA-derived red-emitting fluorescent MR ligands, comprising compounds **156** and **157** (Figure 4.1). Whereas the indolinium-type cyanine dye containing ligands (e.g. **157**) were investigated in fluorescence-based techniques, the pyridinium-type cyanine dye containing probes (e.g. **156**) were only characterized with respect to M_1R-M_5R affinity in radioligand competition binding studies.[47] In the present study, a new series of fluorescently labelled dibenzodiazepinone-type MR ligands was prepared using various fluorescent dyes (TAMRA, BODIPY630/650, pyridinium-type cyanine dyes Py-1 and Py-5). The probes were

characterized with respect to M_1R-M_5R affinity (radioligand competition binding) and by flow cytometric saturation binding studies, the latter including the reported ligand **156**.



Compd.	Ref.	pK_i or pK_d				
		M_1	M_2	M_3	M_4	M_5
1	f	8.4	9.5	8.0	8.7	-
151	a	7.2	-	-	-	-
152	b	8.3	8.1	8.0	8.2	7.8
153	c, d	9.3	8.8	-	-	-
154	c, d	10.5	10.4	-	-	-
155	e	6.0	-	-	-	-
156	g	7.9	8.5	6.8	8.0	6.4
157	g	8.3	9.1	6.8	8.4	6.8

Figure 4.1. Structures and MR binding data of the reported fluorescent probes **151**-**155**, being non-selective MR ligands (**151**-**154**) or showing a slight preference for the M_1R (**152**),[33] and structures and MR affinities of the M_2R preferring dibenzodiazepinone-type MR ligands DIBA and the DIBA-derived fluorescent probes **156** and **157**.[47] (note: binding data for all MR subtypes were not reported in all cases). [a]Tahtaoui et al. (reported K_i values were converted to pK_i values);[32] [b]Jones et al. (reported K_i values were converted to pK_i values);[33] [c]Hern et al. (reported K_i values were converted to pK_i values);[34] [d]Nenasheva et al.;[35] [e]Daval et al.;[37] [f]Gitler et al. (reported K_i values were converted to pK_i values);[42] [g]She et al.[47]

4.2. Results and Discussion

4.2.1. Chemistry

A set of six fluorescent dibenzodiazepinone-type MR ligands (**164**-**169)** was prepared from the previously reported amine-functionalized dibenzodiazepinone derivatives **158**[43] and **72**[46] using four different fluorescent dyes: 5-TAMRA succinimidyl ester (**160**), BODIPY 630/650 succinimidyl ester (**161**), and the pyrylium dyes Py-1 (**162**)[48] and Py-5 (**163**)[48] (*cf.* Figure 4.2). Treatment of **158** with the fluorescent dyes **160-163** in the presence of DIPEA

or triethylamine yielded the fluorescent ligands **164**-**167** (Scheme 4.1). Likewise, treatment of **72** with **162** or **163** gave the fluorescent probes **168** and **169**, respectively (Scheme 4.1). Purification by preparative RP-HPLC afforded **164-169** with high purities (≥ 96%, HPLC analysis at 220 nm). All fluorescent ligands were investigated with respect to their chemical stability in PBS pH 7.4. Whereas compounds **164**, **165**, **168** and **169** showed no decomposition within the incubation period of 48 h (Figure S2, 4.6 Supplementary Information), compounds **166** and **167** showed minor decomposition after 24 h (after 24 h, peak areas (220 nm) of **166** and **167** amounted to 94% and 90%, respectively, of the total peak area).

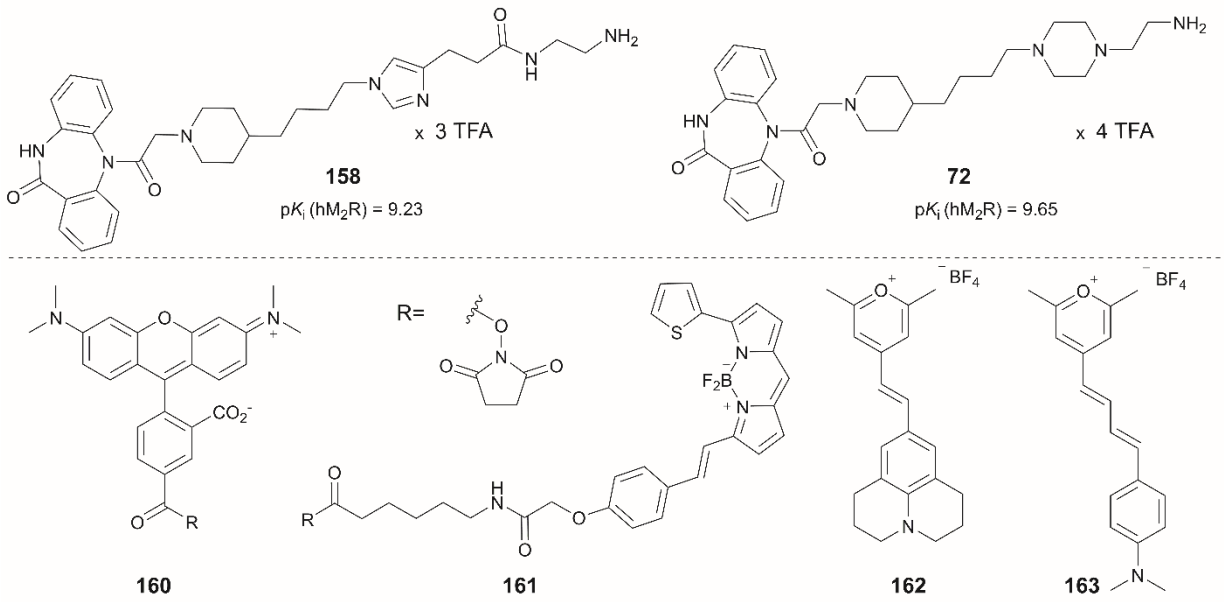
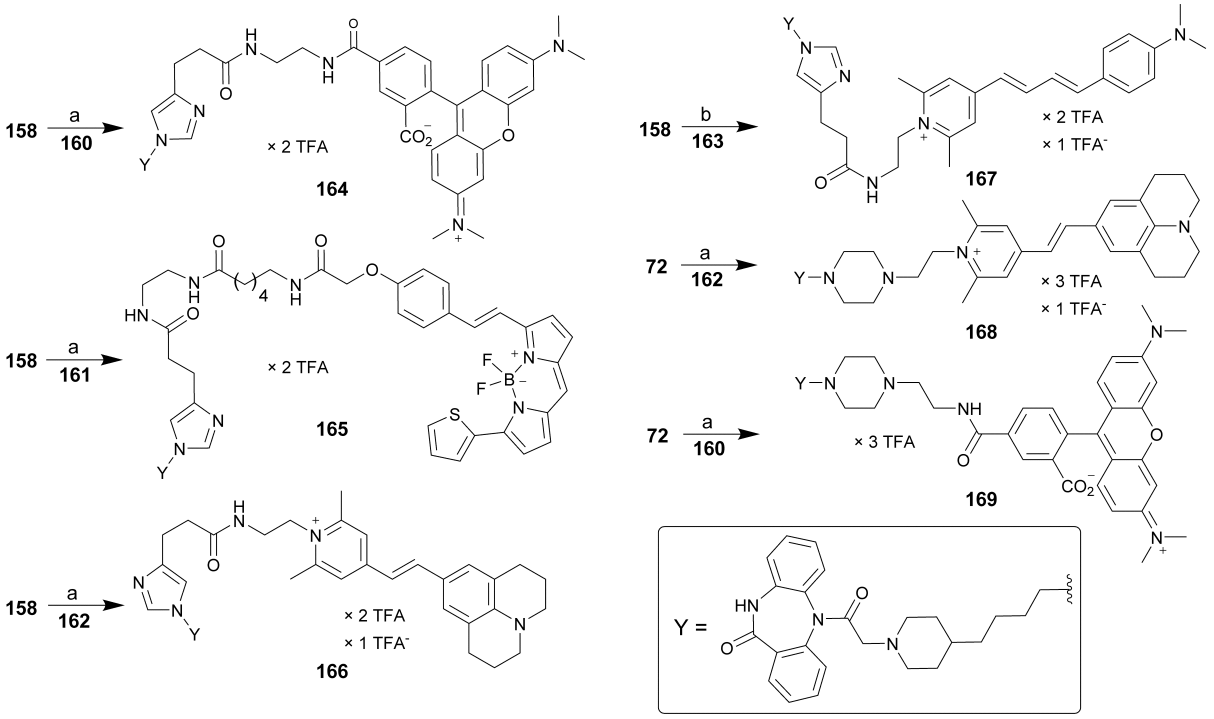


Figure 4.2. Structures and M_2R affinities of the previously described DIBA derived amine-functionalized precursors **158** and **72**,[43, 46] and structures of the fluorescent dyes **160**-**163**, which were used for the preparation of the fluorescent MR ligands **164**-**169** (note: M_2R binding data of **158**, reported as pIC_{50} value,[43] were re-analyzed to obtain the pK_i value).



Scheme 4.1. Synthesis of the fluorescent dibenzodiazepinone-type MR ligands **164-169**, labelled with TAMRA (**164**, **169**), BODIPY630/650 (**165**), pyrylium/pyridinium dye Py-1 (**166**, **168**) or Py-5 (**167**). Reagents and conditions: (a): DIPEA, DMF, rt, 2 h, 31% (**164**), 23% (**165**), 16% (**166**), 23% (**168**), 66% (**169**); (b) triethylamine, DMF, rt, 2h, 28% (**167**).

4.2.2. Radioligand competition binding studies with [^{3}H]NMS

M_1R-M_5R affinities of **164-169** were determined at intact CHO-hM_xR cells (x = 1-5) using the orthosteric non-selective MR antagonist [^{3}H]NMS as radioligand. Compounds **164-169** were capable of completely displacing [^{3}H]NMS from all MR subtypes (Figure 4.3 and Figure S1, 4.6 Supplementary Information). The corresponding pK_i values are listed in Table 1. All fluorescent ligands **164-169** exhibited high M_2R affinity (pK_i values > 8.7). The TAMRA-labelled compound **169** showed the highest M_2R affinity (pK_i: 9.62). For this fluorescent ligand, sigmoidal [^{3}H]NMS displacement curves (M_1R-M_5R) are shown in Figure 4.3B. As in the case of the recently reported series of dibenzodiazepinone-type fluorescent ligands including **156** and **157**,[47] **164-169** showed no or very low preference for the M_2R compared to the M_1 and M_4 receptor, but moderate to pronounced M_2R selectivity towards the M_3 and M_5 subtypes (Table 4.1). Compound **169** exhibited the highest, but still moderate preference for the M_2R over the M_1R and M_4R.

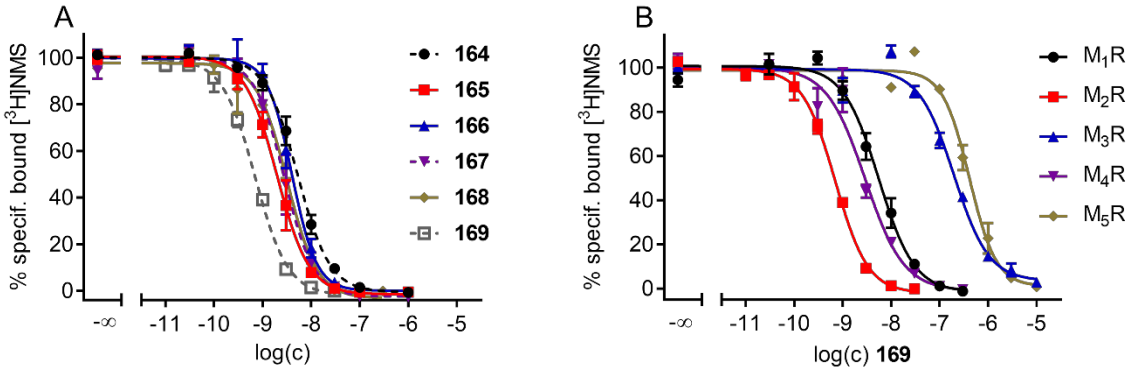


Figure 4.3. (A) Concentration-dependent effects of **164**-**169** on [^{3}H]NMS (c = 0.2 nM) equilibrium M_2R binding determined at intact CHO-hM_2R cells. (B) Radioligand displacement curves obtained from competition binding experiments with [^{3}H]NMS (0.2 nM (M_1R, M_2R, M_3R), 0.1 nM (M_4R) or 0.3 nM (M_5R)) and compound **169** performed at intact CHO-hMxR cells (x = 1-5). M_2R data are the same as in A. Data in A and B represent mean values ± SEM from two (**169**, M_5R) or at least three independent experiments (each performed in triplicate).

Table 4.1. M_1-M_5 receptor binding data (pK_i values) of compounds **156** and **164**-**169** obtained from radioligand competition binding studies, and M_2R binding data (pK_d values) from flow cytometric saturation binding experiments.

compd.	pK_i[a]					pK_d[b]
	M_1R	M_2R	M_3R	M_4R	M_5R	M_2R
156[47]	7.86	8.52	6.83	8.02	6.41	8.63 ± 0.08
164 (UR-CG072)	8.21 ± 0.02	8.75 ± 0.07	6.89 ± 0.06	8.43 ± 0.07	6.56 ± 0.10	8.36 ± 0.09
165 (UR-CG073)	9.00 ± 0.13	9.16 ± 0.10	7.43 ± 0.13	9.38 ± 0.05	8.00 ± 0.08	8.41 ± 0.10
166 (UR-CG074)	8.15 ± 0.05	8.87 ± 0.08	7.16 ± 0.01	8.58 ± 0.08	7.39 ± 0.07	8.70 ± 0.04
167 (UR-AP175)	8.31 ± 0.04	9.04 ± 0.15	7.07 ± 0.04	8.37 ± 0.04	7.15 ± 0.06	8.74 ± 0.13
168 (UR-CG135)	8.32 ± 0.03	9.02 ± 0.10	7.20 ± 0.15	8.57 ± 0.01	6.90 ± 0.03	9.19 ± 0.03
169 (UR-MK342)	8.59 ± 0.10	9.62 ± 0.04	7.09 ± 0.06	9.01 ± 0.03	6.75 ± 0.05	8.86 ± 0.06

[a]Determined by competition binding with [^{3}H]NMS (K_d values/applied concentrations: M_1, 0.17/0.2 nM; M_2, 0.10/0.2 nM; M_3, 0.12/ 0.2 nM; M_4, 0.052/0.1 nM; M_5, 0.20/0.3 nM) at whole CHO-hM_xR cells (x = 1-5) at 23 °C. Means ± SEM from two (**169**, M_5R) or at least three independent experiments (each performed in triplicate). [b]Determined by flow cytometric saturation binding experiments at intact CHO-hM_2R cells at 22 °C. Means ± SEM from two (**169**) or at least three independent experiments (performed in duplicate).

4.2.3. Fluorescence properties

Excitation and corrected emission spectra of **164**-**169**, recorded in PBS containing 1% bovine serum albumin (BSA), are shown in supplementary Figure S3 (4.6. Supplementary Infromation) and the respective excitation and emission maxima are summarized in Table 4.2. It should be noted that compounds **156** and **167**, both labelled with the pyrylium/pyridinium-type fluorescent dye Py-5 are perfectly suited for an excitation with an argon laser (488 nm), belonging to the standard equipment of many instruments. Compound **165** is well suited for an excitation with the commonly used red diode laser (ca. 635 nm), and **164**, **166**, **168** and **169** require green light for an optimal excitation (*cf*. Table 4.2).

Table 4.2. Excitation and emission maxima of the fluorescent ligands **156**, **164-169** determined in PBS containing 1% BSA.

compd.	dye	$\lambda_{ex}/\lambda_{em}$
156[47]	pyridinium cyanine (Py-5)	484/643
164	5-TAMRA	557/583
165	BODIPY 630/650	639/647
166	pyridinium cyanine (Py-1)	526/602
167	pyridinium cyanine (Py-5)	483/634
168	pyridinium cyanine (Py-1)	526/604
169	5-TAMRA	550/583

4.2.4. Flow cytometric M_2R saturation binding studies

M_2R affinities of the fluorescent ligands **156** and **164-169** were also determined by flow cytometric saturation binding studies at intact CHO-hM_2R cells at 22 °C using a FACSContoII (**156**, **164-169**) or a FACSCalibur (**169**) flow cytometer, both equipped with two light sources (argon and red diode laser) (*cf*. Figure 4.4). For these experiments, an incubation period of 2 h was applied because association binding experiments with recently reported radio- and fluorescence labelled dibenzodiazepinone-type MR ligands at intact CHO-hM_2R cells or cell homogenates revealed that equilibrium was reached within 2 h.[45-47] The obtained pK_d values of compounds **156** and **164-169** were in good agreement with the corresponding pK_i values obtained from competition binding studies with [^{3}H]NMS (Table 4.1).

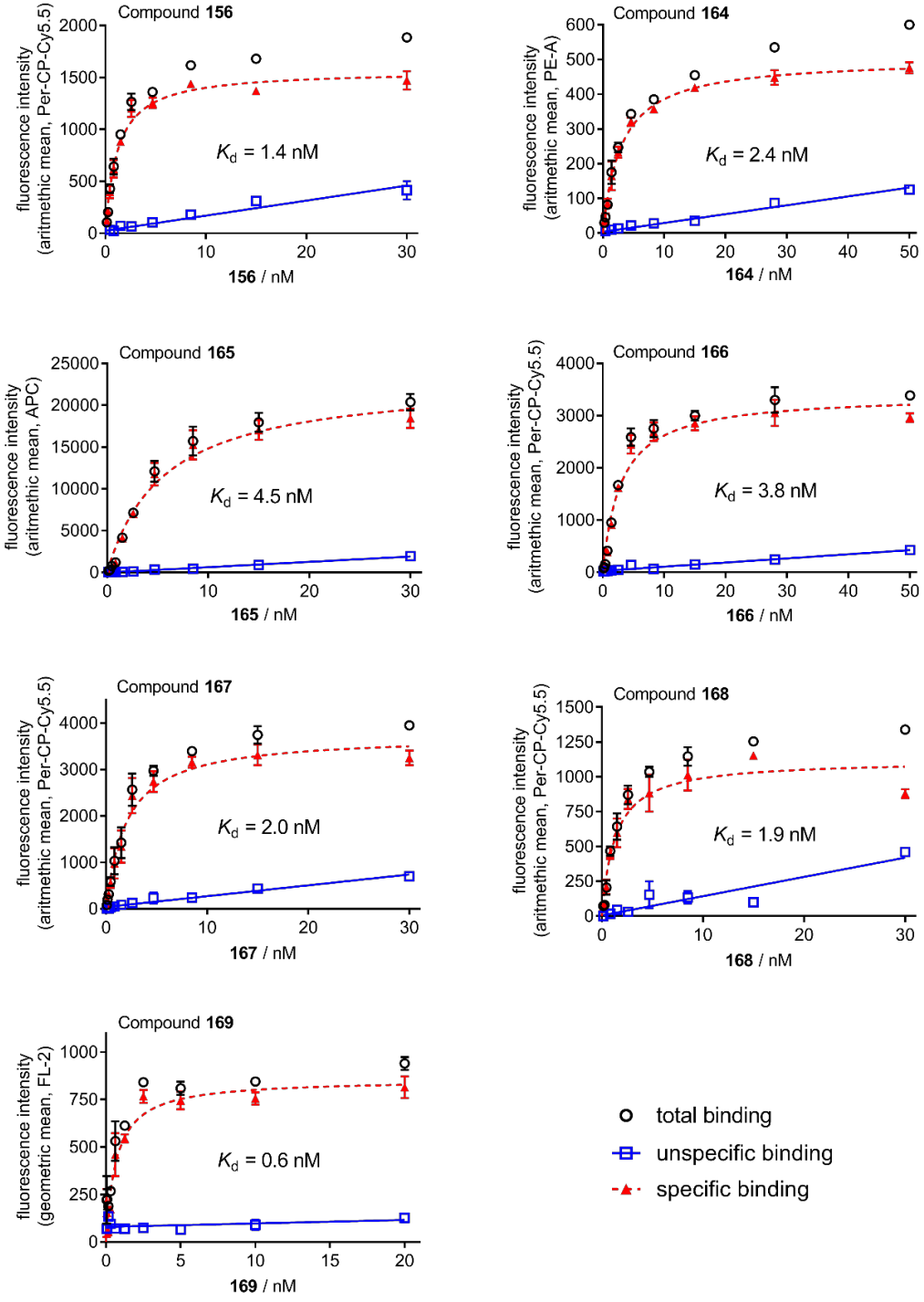


Figure 4.4. Representative saturation isotherms (specific binding, dashed line) obtained from flow cytometric saturation binding experiments performed with **156** and **164-169** at intact CHO-hM_2R cells. Unspecific binding was determined in the presence of atropine (for structure see Figure S4, 4.5. Supplementary Information) used in 500- or 1000-fold excess. Cells were incubated with the fluorescent ligands at 22 °C in the dark for 2 h. Experiments were performed in duplicate. Measurements were performed on a FACSCantoII (**156**, **164-168**) or a FACSCalibur (**169**) flow cytometer (Becton Dickinson). Used laser lines/emission filters: **164**, 488 nm/585 ± 21 nm (PE channel); **165**, 488 nm/660 ± 10 nm (APC channel); **156** and **166-168**, 488 nm/670 ± 65 nm (PerCP-Cy5 channel); **169**, 488 nm/585 ± 21 nm (channel FL-2). Data represent mean values ± SEM (total and unspecific binding) or calculated values ± propagated error (specific binding). Note: total and unspecific binding data represent autofluorescence-corrected data.

4.3. Conclusion

The synthesized series of fluorescence-labelled dibenzodiazepinone-type M_2R ligands represents an extension of a recently reported set of fluorescent MR ligands[47] with respect to the type of attached fluorescent dyes. As the 5-TAMRA fluorophore exhibits a fluorescence life-time (ca. 2.4 ns), which is well compatible with fluorescence anisotropy measurements,[49] the TAMRA-labelled MR ligands (**164**, **169**) will be characterized in fluorescence anisotropy-based assays in future studies including real-time kinetic measurements. Furthermore, the Py-5 labelled probes (**156**, **167**), exhibiting an excitation maximum around 480 nm and a large Stokes shift (emission maximum ≈ 630 nm) should be ideal probes for a recently reported BRET-based GPCR binding assay,[4] requiring fusion of the NanoLuciferase (λ_{max} (bioluminescence) ≈ 460 nm) to the M_2R.

4.4. Experimental section

4.4.1. General experimental conditions

All chemicals and solvents were purchased from commercial suppliers and were used without further purifications. Acetonitrile for HPLC (gradient grade) was obtained from Merck (Darmstadt, Germany) or Sigma-Aldrich (Taufkirchen, Germany). Atropine was purchased from Sigma-Aldrich. The 5-carboxytetramethylrhodamine succinimidyl ester (**160**) was purchased from ABCR (Karlsruhe, Germany) and BODIPY 630/650 succinimidyl ester (**161**) was purchased from Lumiprobe (Hannover, Germany). The pyrylium dyes **158** and **72** were prepared according to described procedures.[48] [^{3}H]NMS (specific activity = 80 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO) via Hartman Analytics (Braunschweig, Germany). Millipore water was used throughout for the preparation of stock solutions and HPLC eluents. Polypropylene reaction vessels (1.5 or 2 mL) with screw cap (Süd-Laborbedarf, Gauting, Germany) were used for the synthesis of all fluorescent ligands and for the preparation and storage of stock solutions. NMR spectra were recorded on a Bruker Avance III HD 600 equipped with a cryogenic probe (14.1 T; ^{1}H: 600 MHz) (Bruker, Karlsruhe, Germany). Abbreviations for the multiplicities of the signals are s (singlet), d (doublet), t (triplet), m (multiplet) and brs (broad singlet). High-resolution mass spectrometry (HRMS) analysis was performed on an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA) using an ESI source. Preparative HPLC of compounds **164**-**169** was performed with a Prep 150 LC system from Waters (Eschborn, Germany) consisting of a binary gradient module, a 2489 UV/visible detector, and a Waters fraction collector III. Compounds **167** and **169** were purified with a preparative HPLC system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. For both systems a Kinetex-XB C18 (5 µm, 250 mm × 21 mm;

Phenomenex, Aschaffenburg, Germany) served as stationary phases at a flow rate of 20 mL/min. Mixtures of 0.1% aq TFA and acetonitrile were used as mobile phase. The detection wavelength was set to 220 nm throughout. The solvent of collected fractions was removed by lyophilization using a Scanvac freeze drying apparatus (Labogene, Allerød, Denmark) equipped with a RZ 6 rotary vane vacuum pump (Vacuubrand, Wertheim, Germany). Analytical HPLC analysis was performed with a system from Agilent Technologies composed of a 1290 Infinity binary pump equipped with a degasser, a 1290 Infinity autosampler, a 1290 Infinity thermostated column compartment, a 1260 Infinity diode array detector, and a 1260 Infinity fluorescence detector. A Kinetex-XB C18 (2.6 µm, 100 × 3 mm; Phenomenex) was used as stationary phase at a flow rate of 0.6 mL/min. Mixtures of 0.04% aq TFA (A) and acetonitrile (B) were used as mobile phase. The following linear gradients were applied: compounds **164**-**166**, **168** and **169** (purity): 0-12 min: A/B 90:10 to 55:45, 12-16 min: 55:45 to 5:95, 16-20 min: 5:95; compound **167** (purity): 0-15 min: A/B 90:10 to 50:50, 15-19 min: 50:50 to 5:95; 20-22 min: 5:95; compounds **164**-**169** (chemical stabilities): 0-20 min: A/B 90:10 to 35:65, 20-22 min: 35:65 to 5:95, 22-26 min: 5:95. For all analytical HPLC runs the oven temperature was set to 25 °C, the injection volume was 20 µL and detection was performed at 220 nm. The stock solutions (final concentration: 1 mM) of fluorescent ligands were prepared in DMSO and were stored at -78°C.

Annotation concerning the ^{1}H-NMR spectra of the dibenzodiazepinone derivatives **164**-**169**: due to a slow rotation about the exocyclic amide group of the diazepinone ring on the NMR time scale, two isomers (ratios provided in the experimental protocols) were evident in the ^{1}H-NMR spectra.

4.4.2. Compounds characterization

All target compounds (**164**-**169**) were characterized by ^{1}H-NMR spectroscopy, HRMS, and RP-HPLC analysis. The HPLC purity of all fluorescent ligands amounted to ≥ 96% (220 nm) (chromatograms shown in the Supplementary Information, NMR spectra shown in Appendix).

4.4.3. Experimental synthetic protocols and analytical data

2-(6-(Dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-5-((2-(3-(1-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)-1*H*-imidazol-4-yl)propanamido)ethyl)carbamoyl)benzoate bis(hydrotrifluoroacetate) (164)

The reaction was carried out in a 1.5-mL propylene reaction vessel equipped with a micro stir bar. Amine precursor **158** (tris(hydrotrifluoroacetate), 14.5 mg, 0.016 mmol) and DIPEA (10.3 mg, 14 µL, 0.089 mmol) were dissolved in anhydrous DMF (90 µL) followed by the addition of **160** (4.2 mg, 0.008 mmol) dissolved in anhydrous DMF (50 µL). After stirring at room temperature in the dark for 2 h, 10% aq TFA (corresponding to 0.09 mmol of TFA) were added. Purification of the product by preparative HPLC (gradient: 0-40 min: 0.1% aq TFA/acetonitrile 81:19-28:72, t_R = 13 min) yielded compound **164** as a red solid (3.0 mg, 31%). Ratio of configurational isomers evident in the ^{1}H-NMR spectrum: ca 1.5:1. ^{1}H-NMR (600 MHz, MeOH-d_4): δ (ppm) 1.33-1.40 (m, 4H), 1.44-1,57 (m, 3H), 1.85-1.93 (m, 3H), 1.93-1.99 (m, 1H), 2.62 (t, 2H, *J* 7.2 Hz), 2.89-2.98 (m, 1H), 3.00 (t, 2H, *J* 7.2 Hz), 3.02-3.10 (m, 1H), 3.45(t, 3H, *J* 6.0 Hz, interfering with the ^{13}C satellite of the solvent residual peak), 3,56 (t, 2H, *J* 6.1 Hz), 3.72 (d, 1.4H, *J* 7.2 Hz), 3.80 (d, 0.60H, *J* 17 Hz), 4.18 (t, 2H, *J* 7.2 Hz), 4.37-4.46 (m, 1H), 7.0-7.02 (m, 2H), 7.03-7.08 (m, 2H), 7.11-7.14 (m, 2H), 7.24-7.36 (m, 2H), 7.36-7.43 (m, 1.4H), 7.46-7.55 (m, 3.2H), 7.59-7.65 (m, 1.4H), 7.65-7.70 (m, 0.6H), 7.72-7.77 (m, 0.4H), 7.87-7.92 (m, 0.6H), 7.95-7.99 (m, 0.4H), 8.22-8.26 (m, 1H), 8.73-8.77 (m, 1H), 8.80 (s, 1H). Note: exchangeable protons (NH, OH) were not apparent. The proton signals of the four methyl groups (12 protons) were not apparent due to an interference with the solvent residual peak. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{57}H_{62}N_9O_7]^+$: 984.4767, found: 984.4757. RP-HPLC (220 nm): 97% (t_R = 7.40 min, *k* = 8.7). $C_{57}H_{61}N_9O_7 \cdot C_4H_2F_6O_4$ (984.17 + 228.04).

(E)-6-(2-(4-(2-(5,5-Difluoro-7-(thiophen-2-yl)-5H-4l4,5l4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-3-yl)vinyl)phenoxy)acetamido)-N-(2-(3-(1-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)-1H-imidazol-4-yl)propanamido)ethyl)hexanamide bis(hydrotrifluoroacetate) (165)

Compound **165** was prepared from **158** (tris(hydrotrifluoroacetate), 8.6 mg, 0.009 mmol) and **161** (3.1 mg, 0.0047 mmol) according to the procedure used for the synthesis of **164**. DIPEA: 6.1 mg, 8.2 µL, 0.047 mmol. Purification of the product by preparative HPLC (gradient: 0-40 min: 0.1% aq TFA/acetonitrile 81:19-19:81, t_R = 22 min) yielded **165** as a blue solid (1.35 mg, 23%). Ratio of configurational isomers evident in the ^{1}H-NMR spectrum: ca 1.5:1. ^{1}H-NMR (600 MHz, MeOH-d_4): δ (ppm) 1.25-1.33 (m, 7H), 1.42-1.51 (m, 2H), 1.52-

1.64 (m, 4H), 1.77-1.93 (m, 4H), 2.16 (t, 2H, *J* 7.2 Hz), 2.52 (t, 2H, *J* 7.2 Hz), 2.81-2.88 (m, 1H), 2.92 (t, 2H, *J* 7.2 Hz), 2.95-3.02 (m, 1H), 3.21-3.25 (m, 4H, interfering with the solvent peak), 3.26-3.28 (m, 3H, interfering with the solvent peak), 3.36-3.41 (m, 1H, interfering with the ^{13}C satellite of the solvent residual peak), 3.66-3.72 (m, 1H); 4.09 (t, 2H, *J* 7.2 Hz), 4.33-4.42 (m, 1H), 4.58 (s, 2H), 6.87 (d, 1H, *J* 4.2 Hz), 7.03-7.07 (m, 2H), 7.15 (d, 2H, *J* 4.2 Hz), 7.19-7.23 (m, 2H), 7.25-7.35 (m, 3H), 7.39 (s, 1H), 7.39-7.42 (m, 0.4H), 7.46-7.51 (m, 1.6H), 7.52-7.66 (m, 7H), 7.66-7.71 (m, 0.6H), 7.71-7.76 (m, 0.4H), 7.89-7.94 (m, 0.6H), 7.95-8.00 (m, 0.4H), 8.11 (d, 1H, *J* 3.9 Hz), 8.72 (s, 1H). Note: exchangeable protons (NH) were not apparent. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{61}H_{68}BF_2N_{10}O_6S]^+$: 1117.5100, found: 1117.5110. RP-HPLC (220 nm): 99% (t_R = 7.62 min, *k* = 9.0). $C_{61}H_{67}BF_2N_{10}O_6S \cdot C_4H_2F_6O_4$ (1117.14 + 228.04).

(E)-2,6-Dimethyl-1-(2-(3-(1-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)-1H-imidazol-4-yl)propanamido)ethyl)-4-(2-(2,3,6,7-tetrahydro-1H,5H-pyrido[3,2,1-ij]quinolin-9-yl)vinyl)pyridin-1-ium bis(hydrotrifluoroacetate) trifluoroacetate (166)

Compound **166** was prepared from **158** (tris(hydrotrifluoroacetate), 28 mg, 0.031 mmol) and **162** (17 mg, 0.043 mmol) according to the procedure used for the synthesis of **164**. DIPEA: 40 mg, 53 µL, 0.31 mmol. Purification of the product by preparative HPLC (gradient: 0-40 min: 0.1% aq TFA/acetonitrile 85:15-38:62, t_R = 23 min) yielded **166** as a red solid (5.4 mg, 16%). Ratio of configurational isomers evident in the ^1H-NMR spectrum: ca 1.5:1. ^1H-NMR (600 MHz, MeOH-d_4): δ (ppm) 1.32-1.39 (m, 4H), 1.41-1.56 (m, 3H), 1.81-1.92 (m, 3H), 1.93-2.00 (m, 5H), 2.60 (t, 2H, *J* 7.3 Hz), 2.75 (t, 4H, *J* 6.6 Hz), 2.82 (s, 6H), 2.89-2.96 (m, 3H), 2.99-3.06 (m, 1H), 3.45 (t, 1H, *J* 13 Hz, interfering with the ^{13}C satellite of the solvent residual peak), 3.62 (t, 2H, *J* 7.2 Hz), 3.69-3.81 (m, 2H), 4.14 (t, 2H, *J* 7.3 Hz), 4.38 (s, 0.4 H), 4.40-4.42 (m, 0.6H), 4.43-4.48 (m, 2H), 6.83 (d, 1H, *J* 16 Hz), 7.13 (s, 2H), 7.25-7.41 (m, 3.6H), 7.47-7.55 (m, 2.4H), 7.61-7.67 (m, 4H), 7.67-7.71 (m, 0.6H), 7.73-7.77 (m, 0.4H), 7.89-7.92 (m, 0.6H), 7.97 (d, 0.4H, *J* 7.6 Hz), 8.79 (s, 1H). Note: exchangeable protons (NH) were not apparent. The proton signals of two methylene groups (4 protons) of the fluorophore were not apparent due to an interference with the solvent residual peak. HRMS (ESI): *m/z* $[M]^+$ calcd. for $[C_{53}H_{63}N_8O_3]^+$: 859.5018, found: 859.5026. RP-HPLC (220 nm): 96% (t_R = 9.5 min, *k* = 12.0). $C_{53}H_{63}N_8O_3^+ \cdot C_2F_3O_2^- \cdot C_4H_2F_6O_4$ (860.14 + 341.07).

4-((1E,3E)-4-(4-(Dimethylamino)phenyl)buta-1,3-dien-1-yl)-2,6-dimethyl-1-(2-(3-(1-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)-1H-imidazol-4-yl)propanamido)ethyl)pyridin-1-ium bis(hydrotrifluoroacetate) trifluoroacetate (167)

Compound **167** was prepared from **158** (tris(hydrotrifluoroacetate), 6.4mg, 0.007 mmol) and **163** (7.7 mg, 0.021 mmol) following the procedure used for the synthesis of **164**, but triethylamine (7.1 mg, 9.8 µL, 0.07 mmol) was used instead of DIPEA. Purification of the product by preparative HPLC (gradient: 0-40 min: 0.1% aq TFA/acetonitrile 81:19-40:60, t_R = 16 min) yielded **167** as a red solid (2.3 mg, 28%). Ratio of configurational isomers evident in the ^{1}H-NMR spectrum: ca 1.5:1. ^{1}H-NMR (600 MHz, MeOH-d_4): δ (ppm) 1.32-1.38 (m, 4H), 1.39-1.55 (m, 3H), 1.82-1.89 (m, 3H), 1.89-1-97 (m, 1H), 2.60 (t, 2H, *J* 7.3 Hz), 2.85 (s, 6H), 2.89-2.95 (m, 3H), 3.02 (s, 7H), 3.44 (t, 1H, *J* 13 Hz, interfering with the ^{13}C satellite of the solvent residual peak), 3.64 (t, 2H, *J* 7.2 Hz), 3.68-3.80 (m, 2H), 4.15 (t, 2H, *J* 7.4 Hz), 4.36-4.45 (m, 1H), 4.50 (t, 2H, *J* 7.1 Hz), 6.57 (d, 1H, *J* 15 Hz), 6.76 (d, 2H, *J* 8.8 Hz), 6.92-6.99 (m, 1H), 7.02 (d, 1H, *J* 15 Hz), 7.25-7.41 (m, 3.6H), 7.44 (d, 2H, *J* 8.9 Hz), 7.47-7.55 (m, 2.4H), 7.61-7.66 (m, 1.6H), 7.66-7.72 (m, 3H), 7.73-7.78 (m, 0.4H), 7.89-7.92 (m, 0.6H), 7.98 (d, 0.4H, *J* 8.4 Hz), 8.79 (s, 1H). Note: exchangeable protons (NH) were not apparent. HRMS (ESI): *m/z* $[M]^+$ calcd. for $[C_{51}H_{61}N_8O_3]^+$: 833.4861, found 833.4879. RP-HPLC (220 nm): 98% (t_R = 8.4 min, *k* = 10.0). $C_{51}H_{61}N_8O_3^+ \cdot C_2F_3O_2^- \cdot C_4H_2F_6O_4$ (834.10 + 341.07).

E)-2,6-Dimethyl-1-(2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)-4-(2-(2,3,6,7-tetrahydro-1H,5H-pyrido[3,2,1-ij]quinolin-9-yl)vinyl)pyridin-1-ium tris(hydrotrifluoroacetate) trifluoroacetate (168)

Compound **168** was prepared from **72** (17 mg, 0.018 mmol), and **162** (10 mg, 0.026 mmol) according to the procedure used for the synthesis of **164**. DIPEA: 23 mg, 31 µL, 0.18 mmol. Purification of the product by preparative HPLC (gradient: 0-40 min: 0.1% aq TFA/acetonitrile 81:19-29:71, t_R = 18 min) yielded **168** as a red solid (5.1 mg, 23%). Ratio of configurational isomers evident in the ^{1}H-NMR spectrum: ca 1.5:1. ^{1}H-NMR (600 MHz, MeOH-d_4): δ (ppm) 1.33-1.57 (m, 7H), 1.68-1.77 (m, 2H), 1.90-2.00 (m, 6H), 2.56-2.63 (m, 2H), 2.75 (t, 4H, *J* 6.3 Hz), 2.80 (s, 6H), 2.92 (t, 3H, *J* 6.7 Hz), 2.98-3.18 (m, 8H), 3.45 (t, 1H, *J* 12 Hz, interfering with the ^{13}C satellite of the solvent residual peak), 3.52-3.59 (m, 2H), 3.70-3.82 (m, 2H), 4.37-4.46 (m, 1H), 4.51 (t, 2H, *J* 6.6 Hz), 6.84 (d, 1H, *J* 16 Hz), 7.13 (s, 2H), 7.24-7.32 (m, 1H), 7.32-7.36 (m, 1H), 7.40 (t, 0.4H, *J* 7.7 Hz), 7.47-7.56 (m, 2.2H), 7.60-7.72 (m, 5H), 7.76 (t, 0.4H, *J* 7.8 Hz), 7.91 (d, 0.6H, *J* 8.2 Hz), 7.98 (d, 0.4H, *J* 8.2 Hz).

Note: exchangeable protons (NH) were not apparent. The proton signals of two methylene groups (4 protons) of the fluorophore were not apparent due to an interference with the solvent residual peak. HRMS (ESI): *m/z* $[M]^+$ calcd. for $[C_{51}H_{64}N_7O_2]^+$: 806.5116, found 806.5121. RP-HPLC (220 nm): 97% (t_R = 9.6 min, *k* = 12.0). $C_{51}H_{64}N_7O_2^+ \cdot C_2F_3O_2^- \cdot C_6H_3F_9O_6$ (807.12 + 455.09).

2-(6-(Dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-5-((2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)carbamoyl)benzoate tris(hydrotrifluoroacetate) (169)

Compound **169** was prepared from **72** (8.9 mg, 0.010mmol) and **160** (4.1 mg, 0.0077 mmol) according to the procedure used for the synthesis of **164**. DIPEA: 11 mg, 14 µL, 0.083 mmol. Purification of the product by preparative HPLC (gradient: 0-40 min: 0.1% aq TFA/acetonitrile 81:19-30:40, t_R = 13 min) yielded **169** as a red solid (6.5 mg, 66%). Ratio of configurational isomers evident in the ^{1}H-NMR spectrum: ca 1.5:1. ^{1}H-NMR (600 MHz, MeOH-d_4): δ (ppm) 1.35-1.43 (m, 4H), 1.44-1.61 (m, 3H), 1.68-1.77 (m, 2H), 1.89-2.02 (m, 2H), 2.61 (brs, 2H), 2.83 (s, 2H), 2.86-3.00 (m, 1H), 3.00-3.12 (m, 4H), 3.45 (t, 2H, *J* 12 Hz, interfering with the ^{13}C satellite of the solvent residual peak), 3.66 (t, 2H, *J* 6.2 Hz), 3.70-3.83 (m, 2H), 4.40 (d, 0.6H, *J* 17 Hz), 4.44 (d, 0.4H, *J* 17 Hz), 7.00 (d, 2H, *J* 2.2 Hz), 7.05 (d, 0.8H, *J* 2.2 Hz), 7.07 (d, 1.2H, *J* 2.3 Hz), 7.13 (d, 2H, *J* 9.4 Hz), 7.25-7.36 (m, 2H), 7.39 (t, 0.4H, *J* 7.8 Hz), 7.47-7.56 (m, 3.2H), 7.60-7.67 (m, 1.4H), 7.70 (t, 0.6H, *J* 7.4 Hz), 7.76 (t, 0.4H, *J* 7.6 Hz), 7.91 (d, 0.6H, *J* 7.8 Hz), 7.98 (d, 0.4H, *J* 7.7 Hz), 8.23-8.28 (m, 1H), 8.77 (s, 1H). Note: exchangeable protons (NH, OH) were not apparent. The proton signals of the four methyl groups (12 protons) and four protons of the piperazine ring (CH_2 groups) were not apparent due to an interference with the solvent residual peak. HRMS (ESI): *m/z* $[M+H]^+$ calcd. for $[C_{55}H_{63}N_8O_6]^+$: 931.4865, found 931.4868. RP-HPLC (220 nm): 99% (t_R = 6.9 min, *k* = 8.1). $C_{55}H_{62}N_8O_6 \cdot C_6H_3F_9O_6$ (931.15 + 342.06).

4.4.4. Investigation of chemical stability

The chemical stabilities of **164**-**169** were investigated in PBS pH 7.4 at 22 ± 1 °C using propylene vessels. The incubation was started by the addition of 10 µL of 1 mM solution of the fluorescent ligand in DMSO to PBS (90 µL) to yield a final concentration of 100 µM. After 0, 24 and 48 h, aliquots (20 µL) were taken and added to 1% aq TFA/acetonitrile (8:2 v/v) (20 µL). The resulting solutions were analyzed by RP-HPLC (analytical HPLC system and conditions see general experimental conditions; t_R: 7.8 min (**164**), 13.5 min (**165**), 9.95 min (**166**), 8.1 min (**167**), 10.0 min (**168**), 7.3 min (**169**)).

4.4.5. Cell culture

CHO-K9 cells, stably transfected with the DNA of human muscarinic receptors M_1-M_5 (obtained from Missouri S&T cDNA Resource Center; Rolla, MO) were cultured in HAM's F12 medium supplemented with fetal calf serum (Biochrom, Berlin, Germany) (10%) and G418 (Biochrom) (750 µg/mL).

4.4.6. Determination of excitation and emission spectra

Excitation and emission spectra of compounds **164**-**169** were recorded in PBS, pH 7.4, containing 1% BSA (Serva, Heidelberg, Germany), at 22 °C with a Cary Eclipse spectrofluorimeter (Varian Inc., Mulgrave, Victoria, Australia) using acryl cuvettes (10 × 10 mm, Ref. 67.755, Sarstedt, Nümbrecht, Germany). The slit adjustments (excitation/emission) were 5/10 nm for excitation spectra and 10/5 nm in case of emission spectra. Net spectra were calculated by subtracting the respective vehicle reference spectrum, and corrected emission spectra were calculated by multiplying the net emission spectra with the respective lamp corrections spectrum (same slit adjustments, etc.).

4.4.7. Radioligand competition binding assay

Equilibrium competition binding studies with [^{3}H]NMS were performed at intact CHO-hM_xR cells (x = 1-5) at 23 ± 1 °C in white 96-wells plates with clear bottom (Corning Life Science, Tewksbury, MA; Corning cat. No. 3610) using Leibovitz's L-15 medium (Gibco, Life Technologies, Darmstadt, Germany) supplemented with 1% BSA (Serva) as binding buffer (in the following referred to as L15 medium). Experiments were performed using a previously described protocol for MR binding studies with [^{3}H]NMS,[43] but the total volume per well was 200 µL, i.e. wells were pre-filled with 180 µL of L15 medium followed by the addition of L15 medium (20 µL) containing [^{3}H]NMS (10-fold concentrated), to determine total binding, or pre-filled with 160 µL of L15 medium followed by the addition of L15 medium (20 µL) containing atropine or the compound of interest (10-fold concentrated) and L15 medium (20 µL) containing [^{3}H]NMS (10-fold-concentrated), to determine unspecific binding and the displacing effect of a compound of interest, respectively. The concentrations of [^{3}H]NMS were 0.2 nM (M_1R, M_2R, M_3R), 0.1 nM (M_4R) or 0.3 nM (M_5R). Samples were incubated in the dark under gentle shaking for 3 h. Prior to the competition binding experiments, the K_d values of [^{3}H]NMS were determined by saturation binding applying the same conditions (buffer, temperature, incubation time, unspecific binding, etc.). The obtained K_d values amounted to 0.17 ± 0.01 nM (M_1R), 0.10 ± 0.01 nM (M_2R), 0.12 ± 0.01 nM (M_3R), 0.052 ± 0.01 nM (M_4R) and 0.20 ± 0.02 nM (M_5R) (mean value ± SEM from at

least four independent determinations performed in triplicate), being in excellent agreement with previously determined K_d values of [^{3}H]NMS.[43]

4.4.8. Flow cytometric saturation binding experiments

Flow cytometric M_2R binding studies were performed with a FACSCantoII flow cytometer (Becton Dickinson, Heidelberg, Germany) (compounds **156** and **164**-**169**) or with a FACSCalibur flow cytometer (Becton Dickinson) (**169**), both equipped with an argon laser (488 nm) and a red diode laser (640 and 635 nm, respectively). Fluorescence signals were recorded using the following instrument settings: compound **164**, excitation: 488 nm, emission: 585 ± 21 nm (PE channel), gain: 385-440 V; compound **165**, excitation: 633 nm, emission: 660 ± 10 nm (APC channel), gain: 480-510 V; compounds **156** and **166**-**168**, excitation: 488 nm, emission: 670 ± 65 nm (PerCP-Cy5.5 channel), gain: 430 V (**156**) or 465-485 V (**166**-**168**); compound **169**, excitation: 488 nm, emission: 585 ± 21 nm (FL-2), gain: 750 V. Measurements were stopped after counting of 10,000 gated events at medium (**156**, **166**-**168**) or high (**169**) flow rate. All samples were prepared and incubated in 1.5 mL reaction vessels (Sarstedt). Cells were seeded in a 175-cm^2 culture flask 5-6 days prior to the experiment. On the day of the experiment, cells were treated with trypsin, detached and suspended in culture medium followed by centrifugation. The cell pellet was re-suspended in Leibovitz's L15 culture medium (Gibco, Life Technologies) supplemented with 1% BSA (Serva) (in the following referred to as L15 medium). The cell density was adjusted to 1 × 10^6 cells/mL. For the determination of total binding, 2 µL of a solution of fluorescent ligand (100-fold concentrated compared to the final concentration) in DMSO/H_2O (1:1 v/v) and 2 µL of DMSO/H_2O (1:4 v/v) were added to 200 µL of the cell suspension. For the determination of unspecific binding (in the presence of atropine at 500-fold access to the fluorescent ligand), 2 µL of a solution of atropine (100-fold concentrated) in DMSO/H_2O (1:4 v/v) were added instead of neat DMSO/H_2O (1:4 v/v) (note: in case of 20, the sample volume was 500 µL, i.e. 5 µL instead of 2 µL of ligand solution was added, and the excess of atropine was 1000-fold). Compound **164** was used at final concentrations of 0.23-50 nM, **169** was used at final concentrations of 0.04-20 nM, compounds **156** and **165**-**168** were used at final concentrations of 0.15-30 nM. Samples were incubated at 22 °C in the dark under gentle shaking for 2 h. All experiments were performed in duplicate.

4.4.9. Data processing

Retention (capacity) factors were calculated from retention times (t_R) according to $k = (t_R - t_0)/t_0$ (t_0 = dead time). Raw data from flow cytometric experiments were processed with FACSDiva Software (Becton Dickinson) (**156**, **164**-**168**) or with FlowJo Software (FlowJo

LLC, Ashland, OR) (**169**) to obtain arithmetic mean values of the areas of the signals detected in the respective channel (FACSCantoII) and geometric mean values of the height of the signals detected in channel FL-2 (FACSCalibur), respectively. Specific binding data from flow cytometric saturation binding experiments, obtained by subtracting unspecific binding data from total binding data, were plotted against the fluorescent ligand concentration and analyzed by a two-parameter equation describing hyperbolic binding (one site-specific binding; GraphPad Prism 5, GraphPad Software, San Diego, CA) in order to obtain K_d and B_{max} values. K_d values of individual experiments were transformed to pK_d values. Unspecific binding data were fitted by linear regression. Data from the radioligand ([^{3}H]NMS) saturation[43] and competition[44] binding assays were processed as reported previously. pIC_{50} values were converted to pK_i values according to the Cheng-Prusoff equation[50] (logarithmic form). Propagated errors were calculated as described previously.[47]

4.5. Supplementary Information

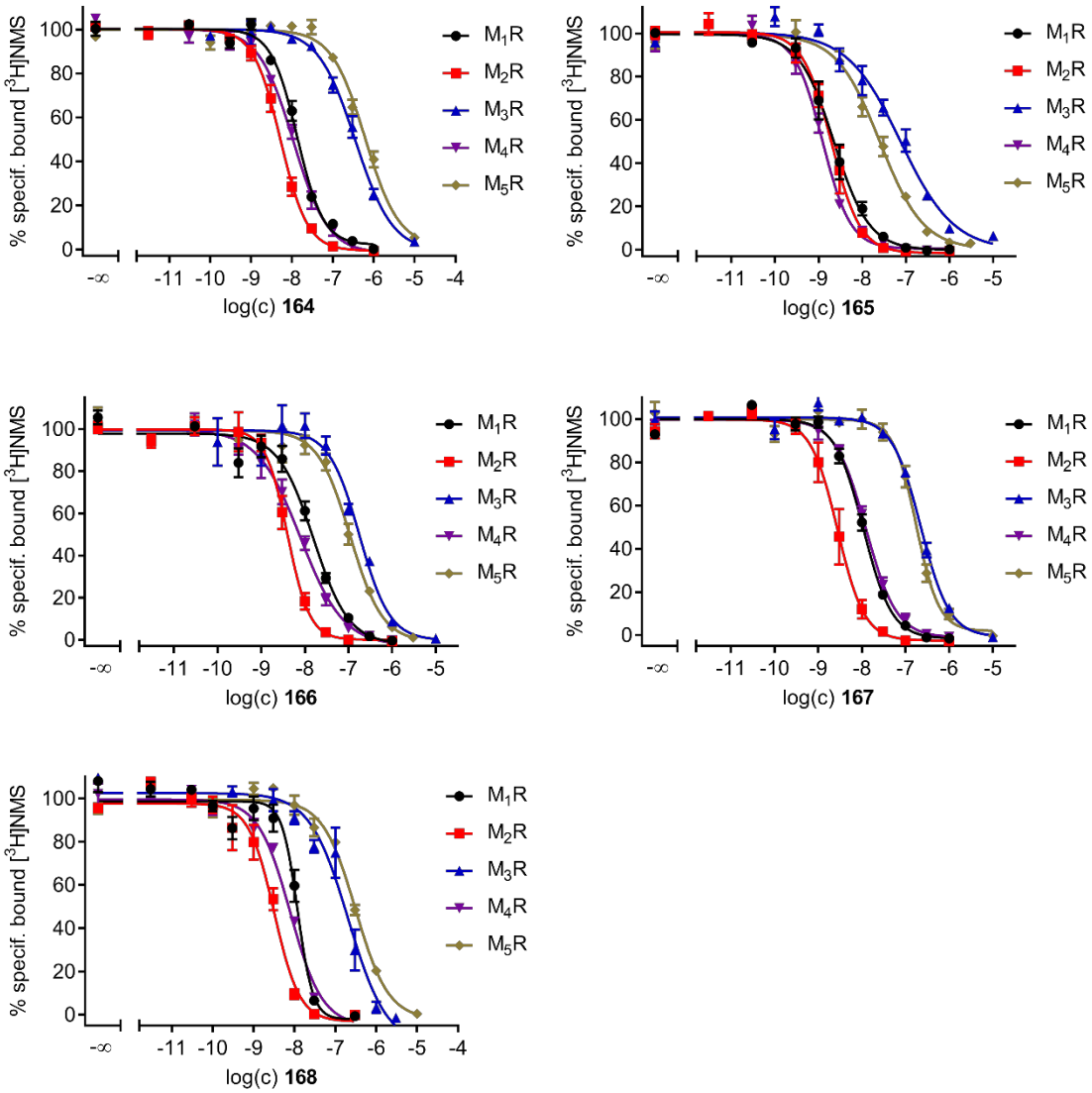


Figure S1. Radioligand displacement curves obtained from competition binding experiments with [^{3}H]NMS (0.2 nM (M_1R, M_2R, M_3R), 0.1 nM (M_4R) or 0.3 nM (M_5R)) and **164-168** at intact CHO-hM_xR cells (x = 1-5). Data represent are mean values ± SEM from at least three independent experiments (each performed in triplicate).

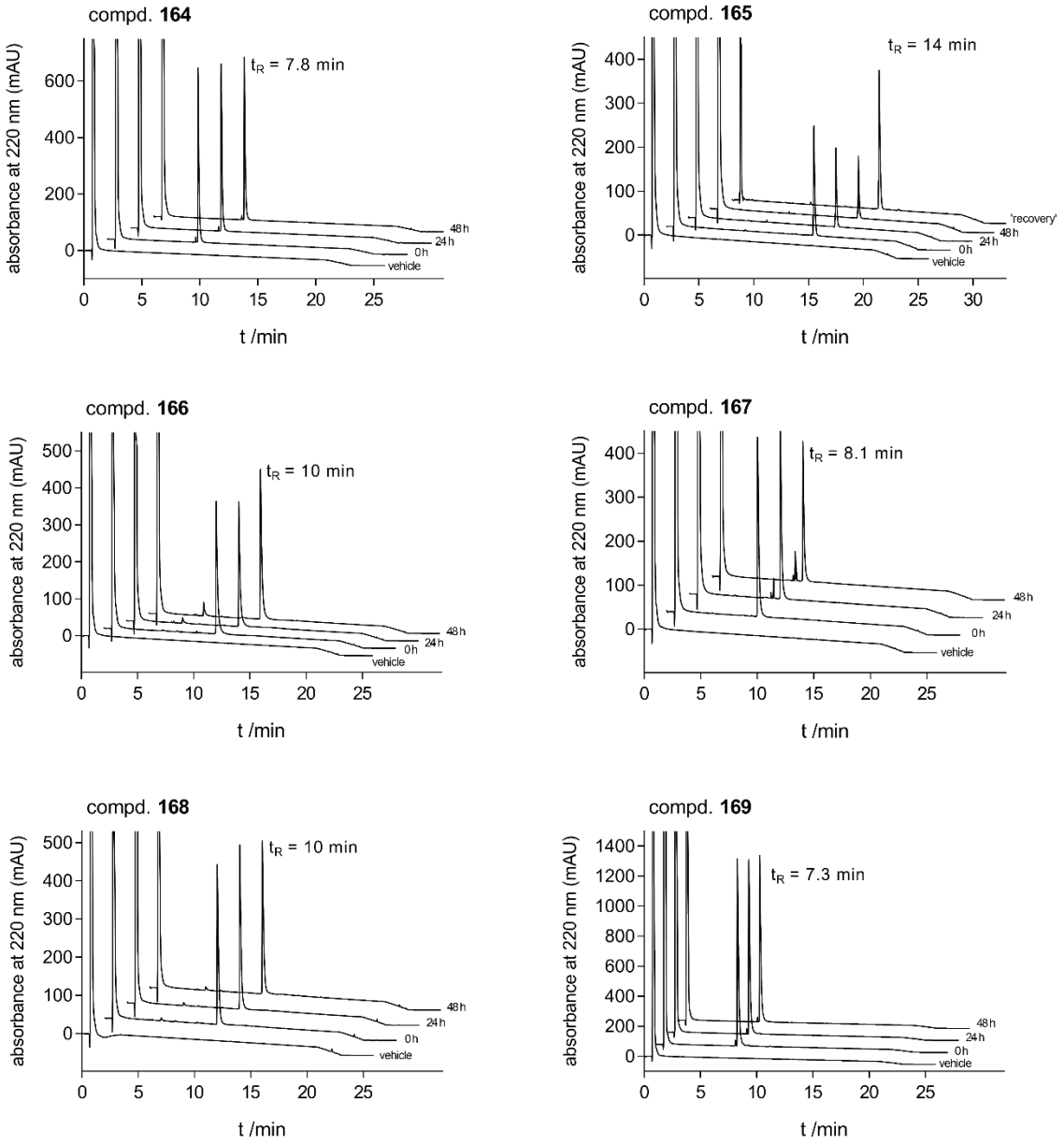


Figure S2. Chromatograms of the HPLC analysis (method see general experimental conditions) of compounds **164**-**169** after incubation in PBS (pH 7.4) at 22 °C for up to 48 h. Whereas compounds **164**, **165**, **168** and **169** showed no decomposition, compounds **166** and **167** showed minor decomposition after 24 h. Compound **165** showed high adsorption to the surface of the polypropylene vessel immediately upon sample preparation. After 48 h, adsorbed **165** was desorbed by replacement of the residual original solution (PBS, pH 7.4) with 0.1% aq TFA/acetonitrile (1:1 v/v, 60 µL) ('recovery'). For injection, this solution was 1:1 diluted with water.

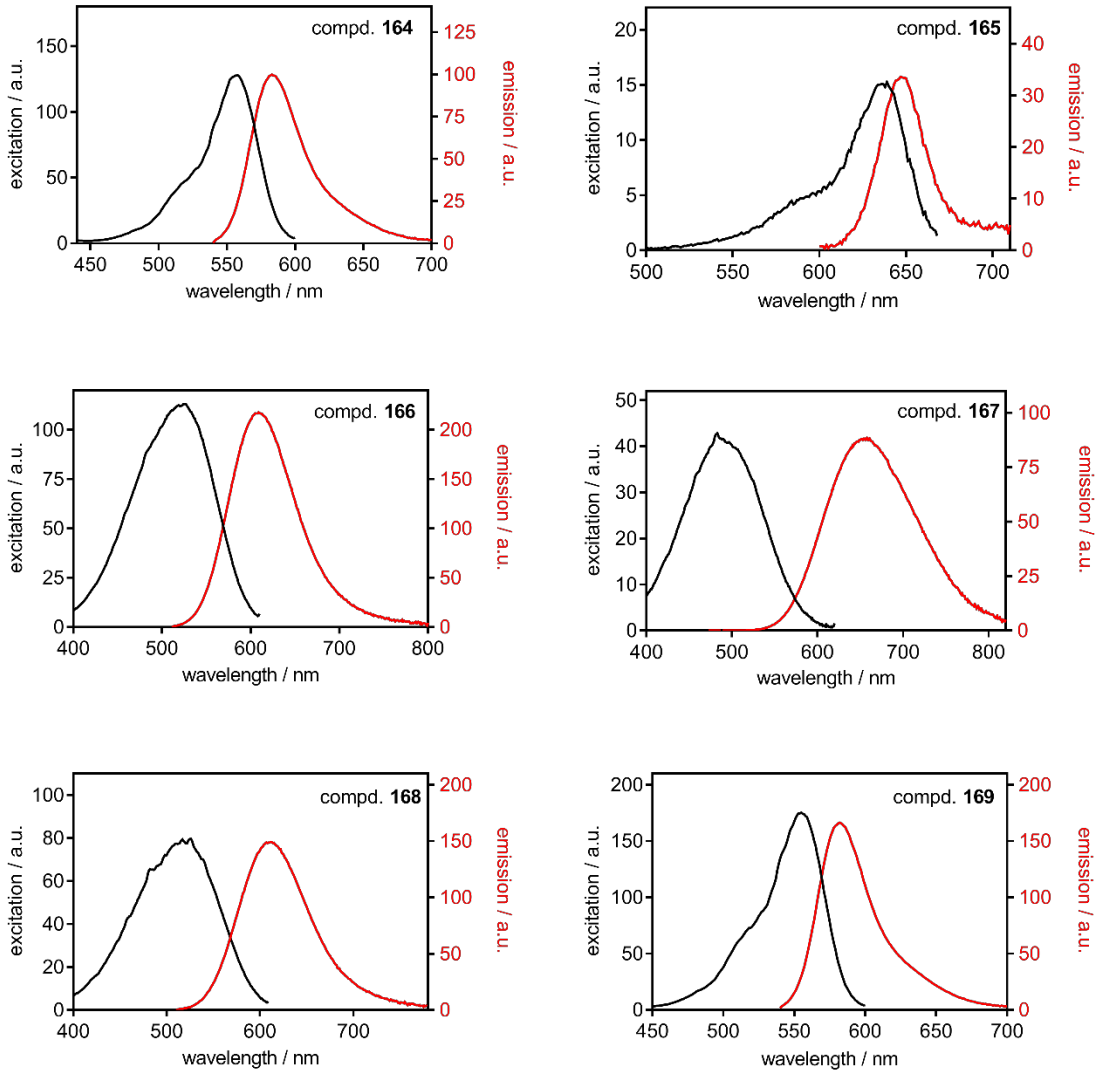
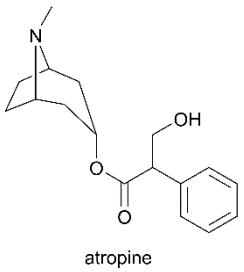


Figure S3. Excitation and corrected emission spectra of fluorescent ligands **164-169** recorded in PBS, pH 7.4, containing 1% BSA, at 22 °C using fluorescent ligand concentrations of 3 µM (**164**, **165**, **169**) or 5 µM (**166**, **167**, **168**).



atropine

Figure S4. Structure of the MR antagonist atropine.

4.6. References

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Summary

In humans, the family of muscarinic acetylcholine receptors (mAChR, MRs) comprises five subtypes (M_1R-M_5R), belonging to class A of the GPCR superfamily. MRs mediate the action of the neurotransmitter acetylcholine (ACh) in the central and peripheral nervous system. Whereas the M_1R, M_3R and M_5R preferably couple to G_q-type G-proteins, the M_2 and M_4 receptor mainly activate $G_{i/o}$-type G-proteins. Due to the high conservation of the orthosteric (acetylcholine) binding site within the family of MRs, the development of highly subtype selective MR ligands is difficult. Selective MR ligands are needed as molecular or pharmacological tools and also as new drug candidates anticipated to cause less adverse effects. As the vestibule of MRs, also referred to as the common allosteric site, is less conserved than the orthosteric pocket, the dualsteric ligand approach, i.e. the design of ligands, which simultaneously address the orthosteric and an allosteric binding site, was suggested as a promising strategy to develop MR ligands with high affinity and improved subtype selectivity.

This work was aiming at the synthesis and pharmacological characterization of dibenzodiazepinone-type MR ligands with pronounced M_2R selectivity. The tricyclic MR ligands were prepared by linking the pharmacophore DIBA *via* various linker moieties to several short peptides, yielding a series of DIBA-peptide conjugates (70 compounds). The linker structure was varied with respect to length, rigidity/flexibility and number of basic groups. All peptide moieties contained at least one basic amino acid. In addition to proteinogenic amino acids, also unnatural amino acids were incorporated. MR affinities and selectivity profiles of the DIBA-peptide conjugates were determined by radioligand competition binding at CHO-hM_xR cells (x = 1-5) using [^{3}H]N-methyl scopolamine as labeled ligand. M_1, M_2 and M_4 receptor affinities were determined for all target compounds. Additionally, M_3 and M_5 receptor affinities were determined for selected compounds, showing either high M_2R selectivity over the M_1 and M_4 receptor, or a very low or no preference for the M_2R.

The study revealed that, besides the peptide structure, the type of the linker considerably determines M_2R affinity and selectivity. DIBA-peptide conjugates derived from amine precursor **35** (compounds **101**-**106**), containing an N-acylated 4-aminopiperidine and a bisalkylated piperazine ring in the linker moiety, exhibited pronounced M_2R selectivity (e.g. **105**: K_i $M_1R/M_2R/M_3R/M_4R/M_5R$ = >140:1:>160:59:>140), but only moderate M_2R affinity (pK_i 7.05-7.67). The subset of compounds derived from amine precursor **40** (**107**-**109**), representing the higher homologue of precursor **35** (extension of the linker by one methylene group), exhibited higher M_2R affinities (pK_i 8.93-9.08) and displayed also high M_2R selectivity (e.g. **109**: K_i $M_1R/M_2R/M_3R/M_4R/M_5R$ = 70:1:11,000:48:6000). Considering M_2R over M_3 and M_5 receptor selectivity, compounds such as DIBA-peptide conjugates **107**-**109** represent the most selective M_2R antagonists reported to date. Regarding M_2R over M_1

and M_4 receptor selectivity, higher M_2R selectivity compared to **103**-**105** or **107**-**109** had only been reported for one other compound class. For three selected DIBA-peptide conjugates, M_2R antagonism was confirmed in a M_2R miniG_i recruitment assay yielding K_b values, which were in excellent agreement with the respective K_i values from radioligand competition binding studies.

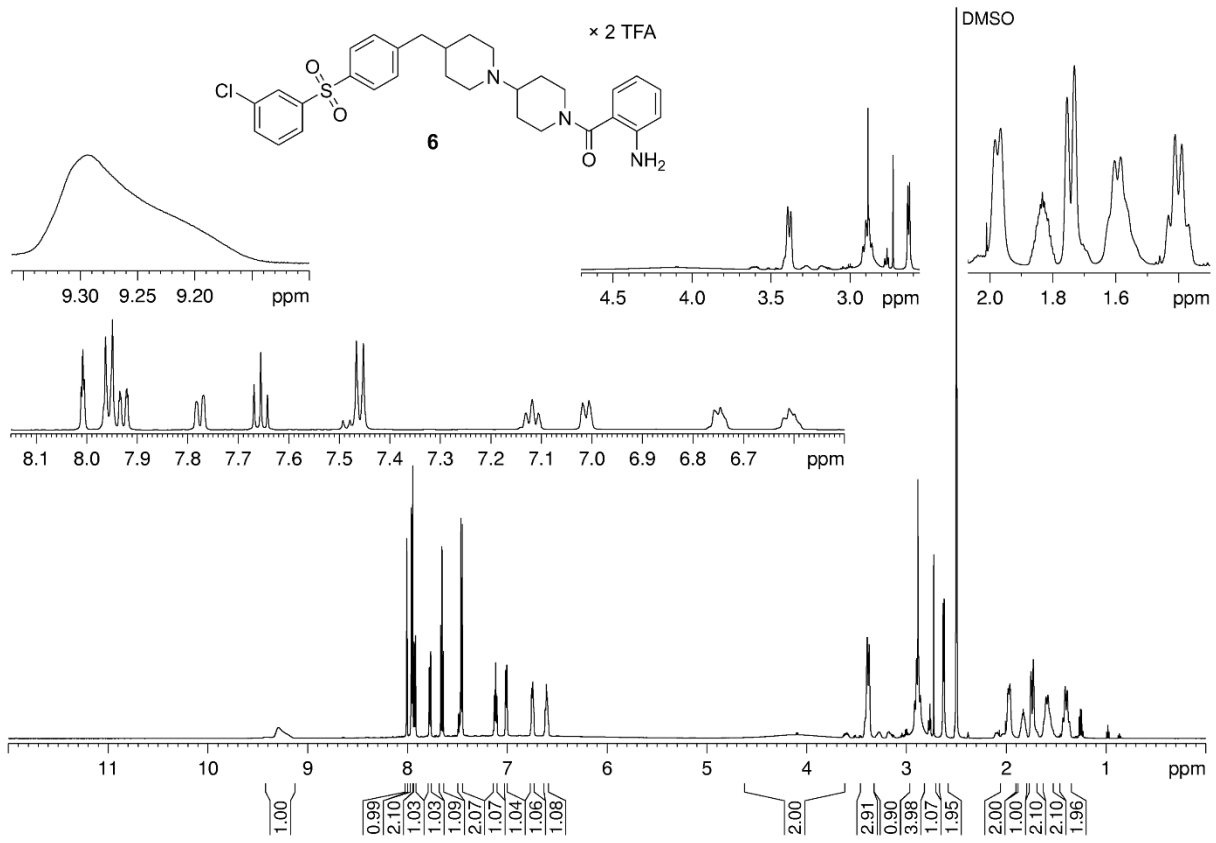
The investigation of three selected DIBA-peptide conjugates (**3**, **4**, **146**) with respect to stability in human plasma showed highly stability against proteolytic degradation (> 99% intact compound after 24 h at 37 °C).

Moreover, six fluorescently labeled dibenzodiazepinone-type MR ligands (**164**-**169**) were prepared using various fluorescent dyes (5-TAMRA, BODIPY, pyridinium dye Py-1, and pyridinium dye Py-5). All fluorescent probes exhibited high M_2R affinity (pK_i (radioligand competition binding): 8.75-9.62), a low preference for the M_2R over the M_1 and M_4 receptor and moderate to pronounced M_2R selectivity compared to the M_3R and M_5R. The fluorescent ligands were successfully used as probes in flow cytometric M_2R saturation binding assays resulting in pK_d values of 8.36-9.19. Therefore, they are considered useful molecular tools for future studies using methods such as fluorescence anisotropy and BRET based MR binding assays.

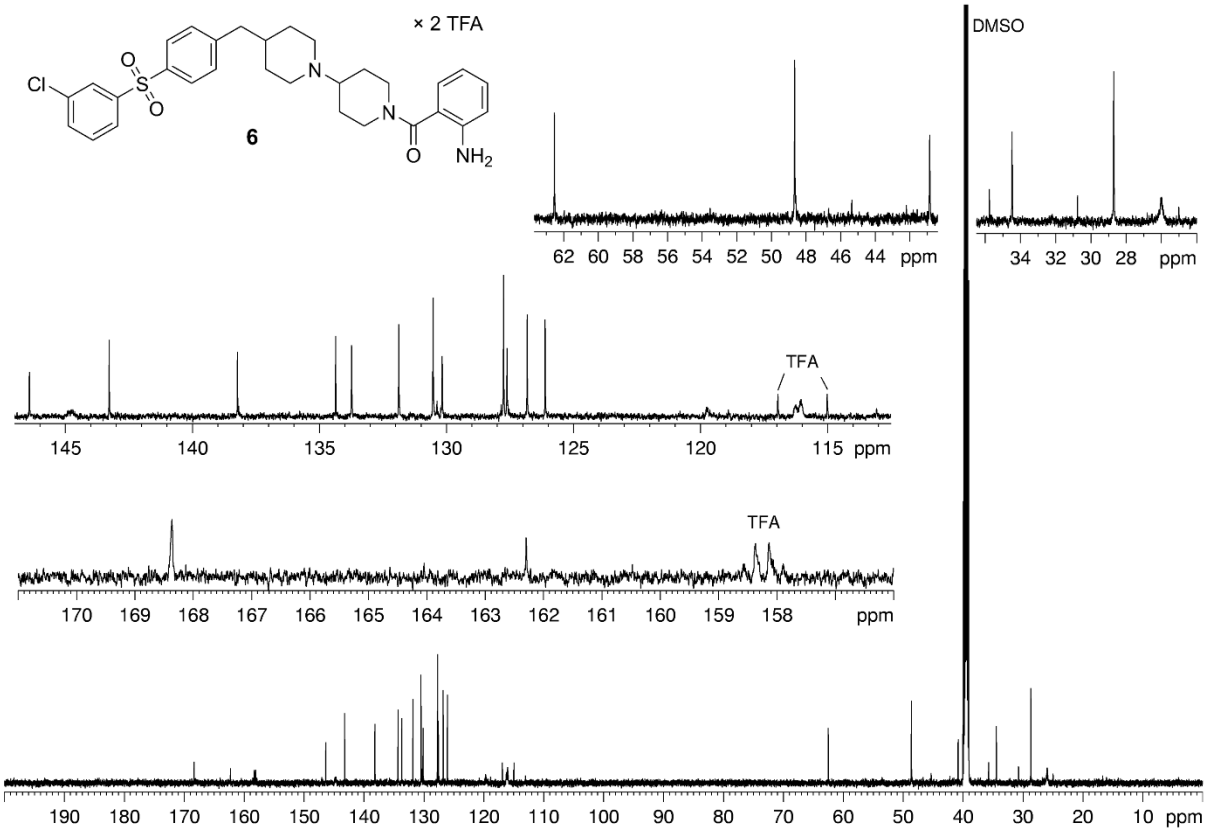
In conclusion, this thesis afforded new fluorescently labeled molecular tools for the M_2R and new highly selective M_2R antagonists, which might serve as lead structures for the development of drug-like selective M_2R antagonists, representing potential therapeutics for the treatment of diseases associated with cholinergic dysfunction such as Alzheimer's disease.

Appendix

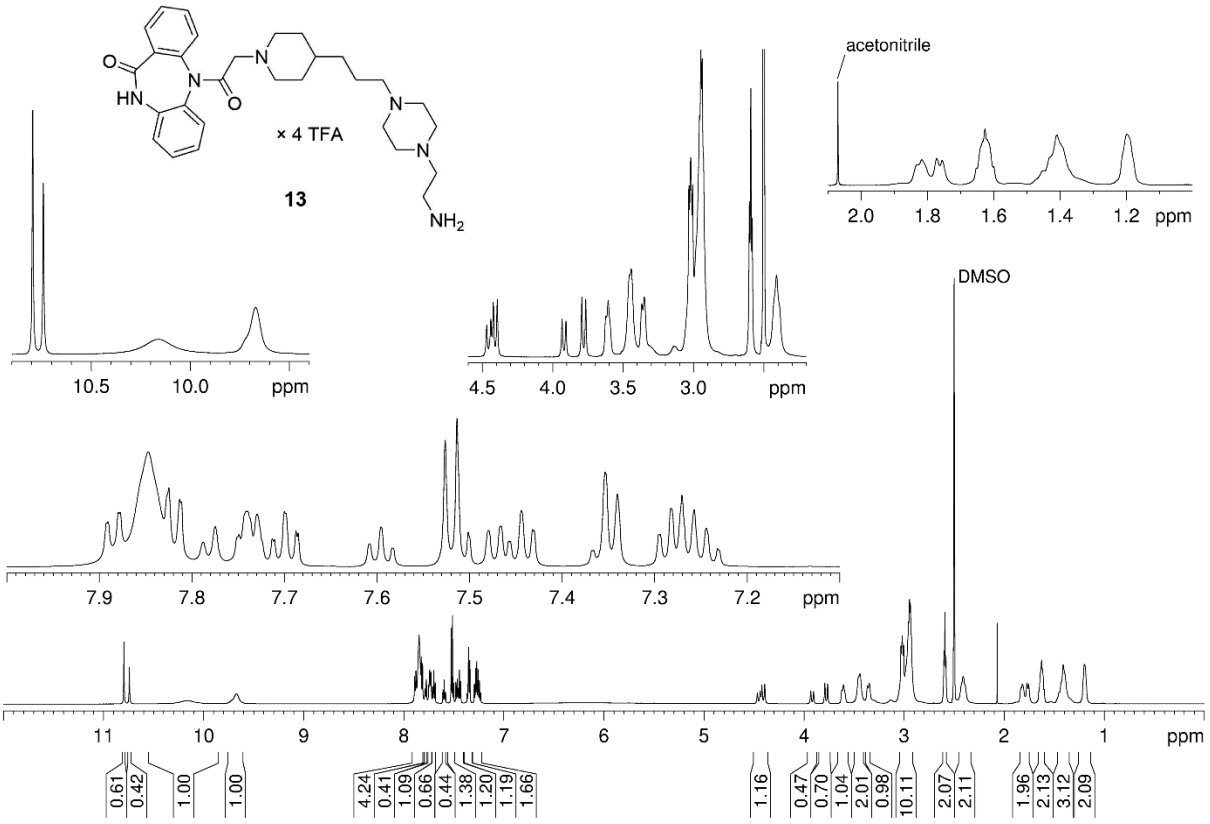
^{1}H and ^{13}C-NMR spectra of compounds 6, 23-25, 28, 31, 35, 40, 43, 53-60, 63-65, 73-82, 85-99, 101-126, 134, 135 and ^{1}H-NMR spectra of compounds 137-139 (Chapter 2)



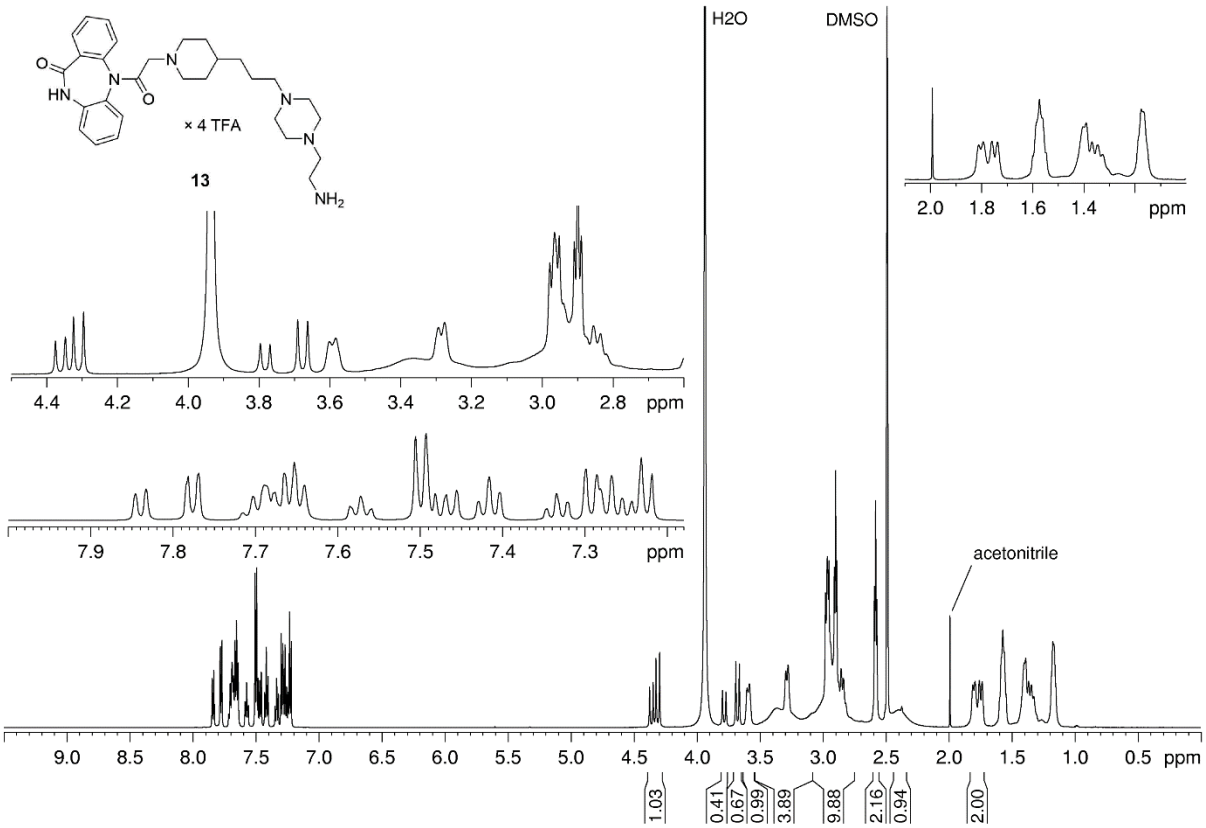
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **6**



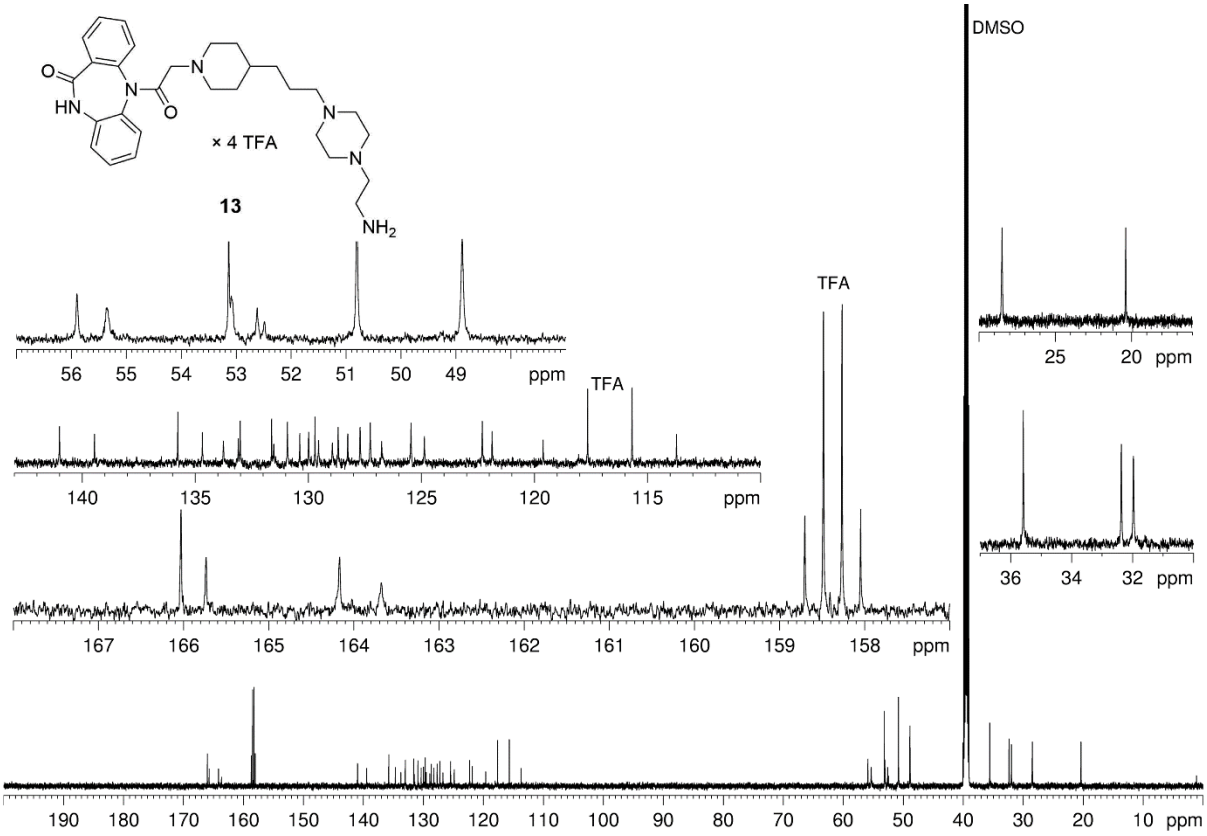
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **6**



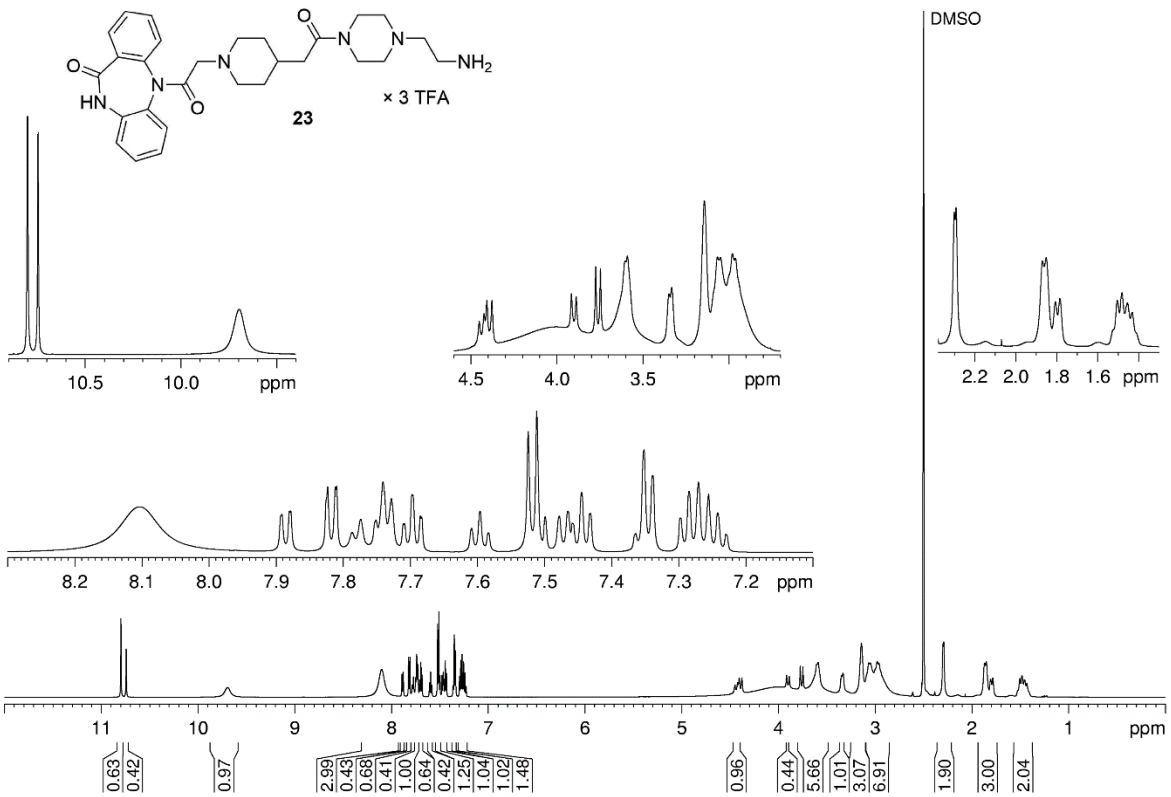
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **13**



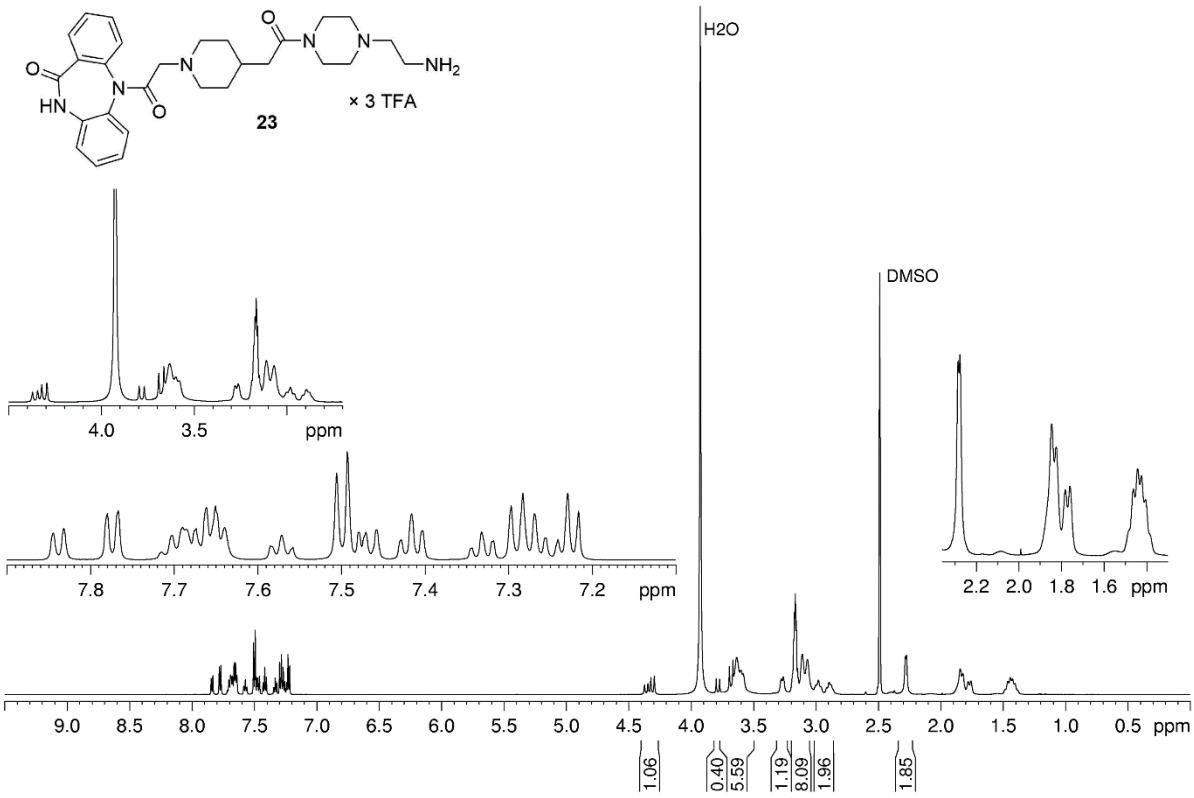
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **13**



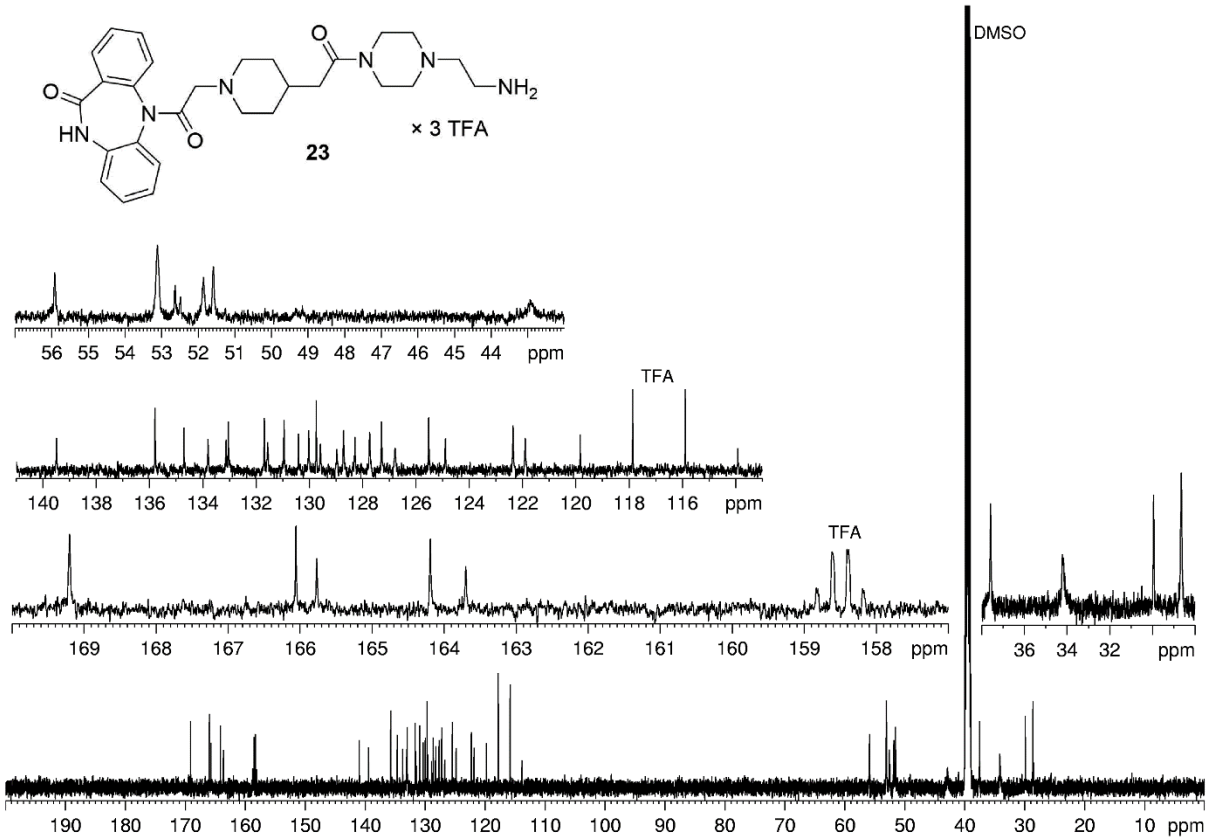
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **13**



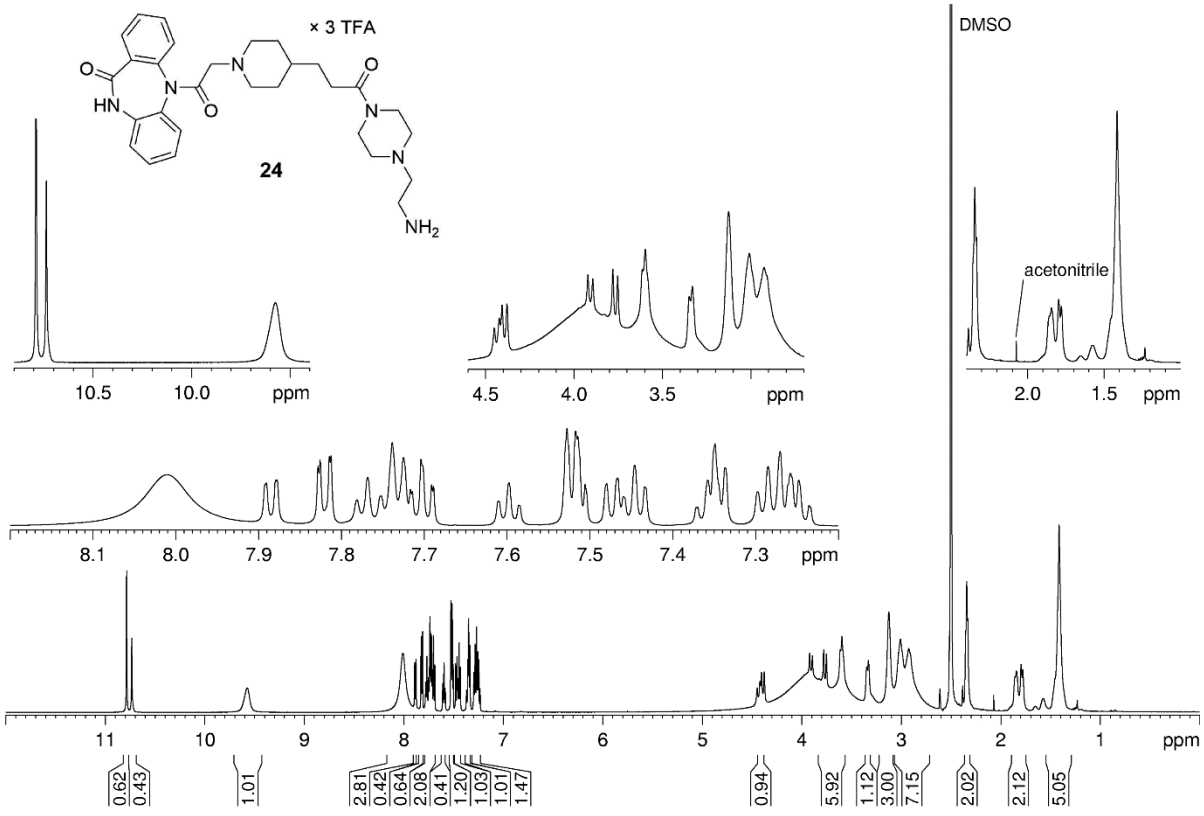
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **23**



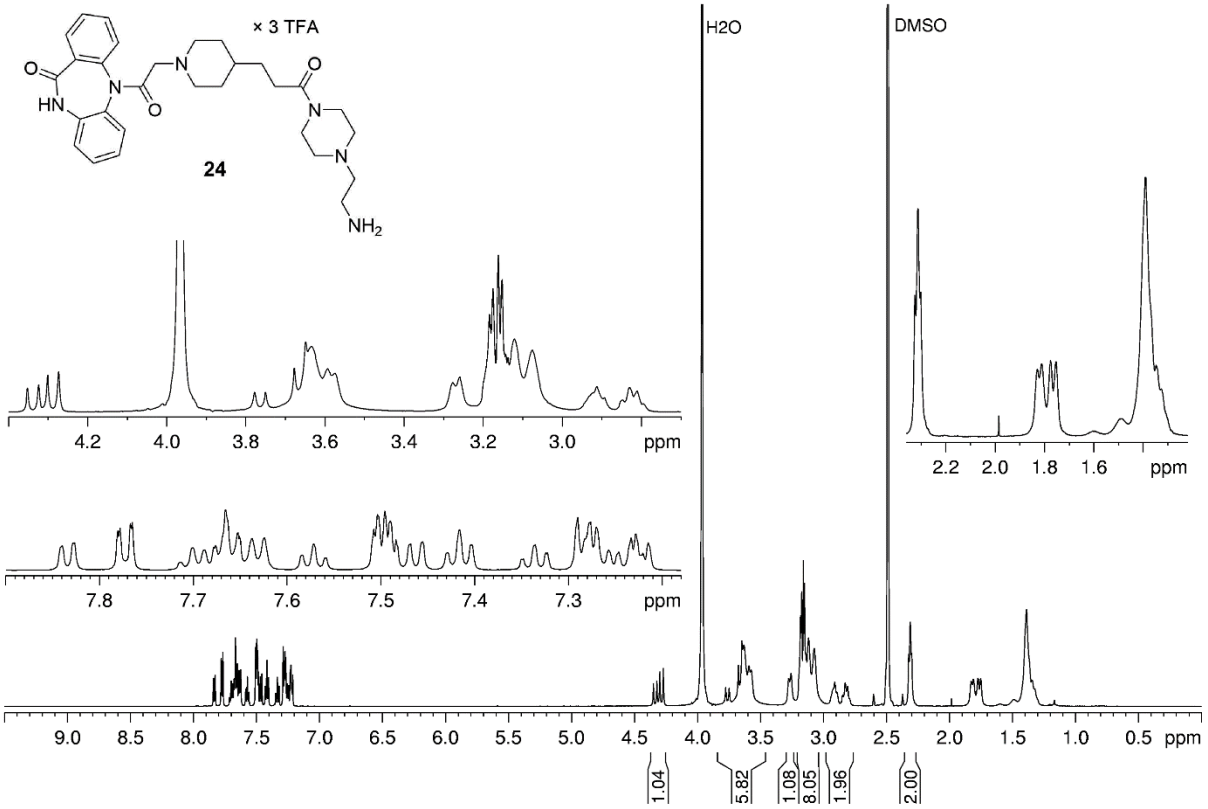
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **23**



^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **23**



^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **24**



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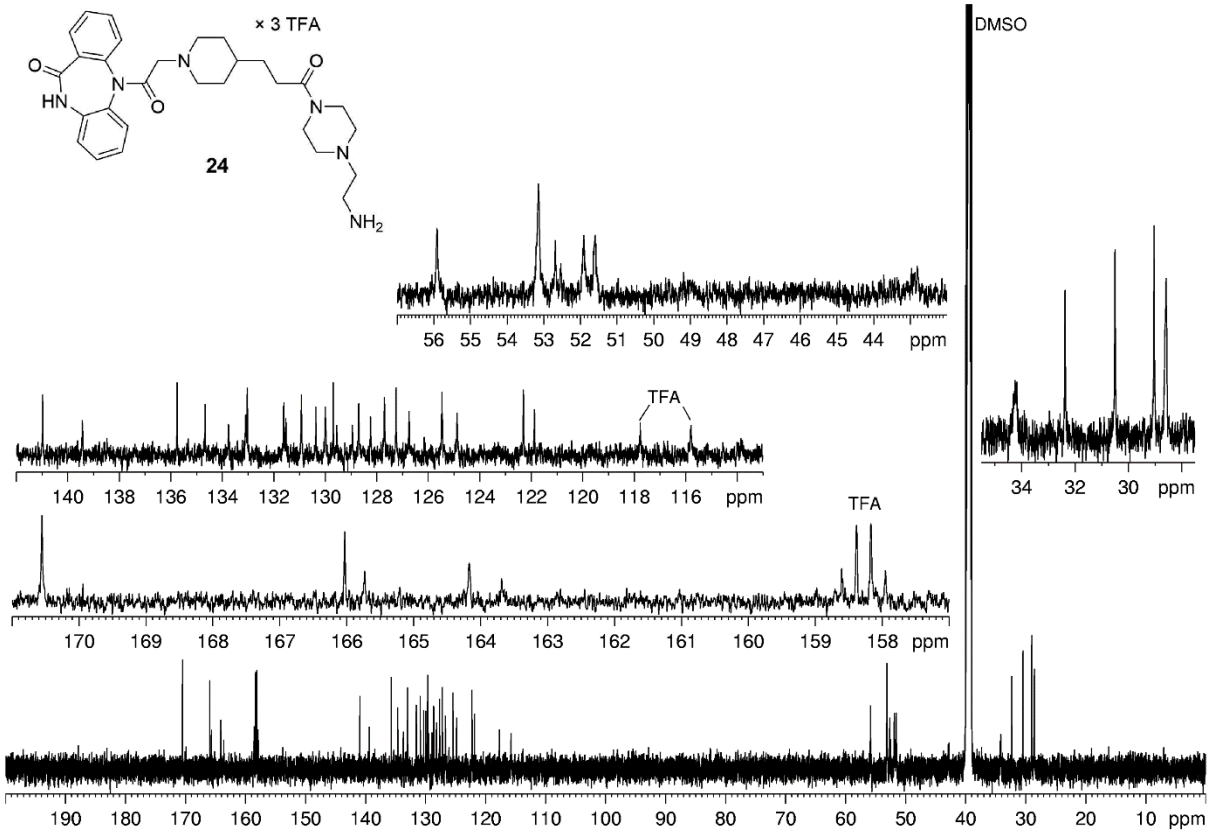
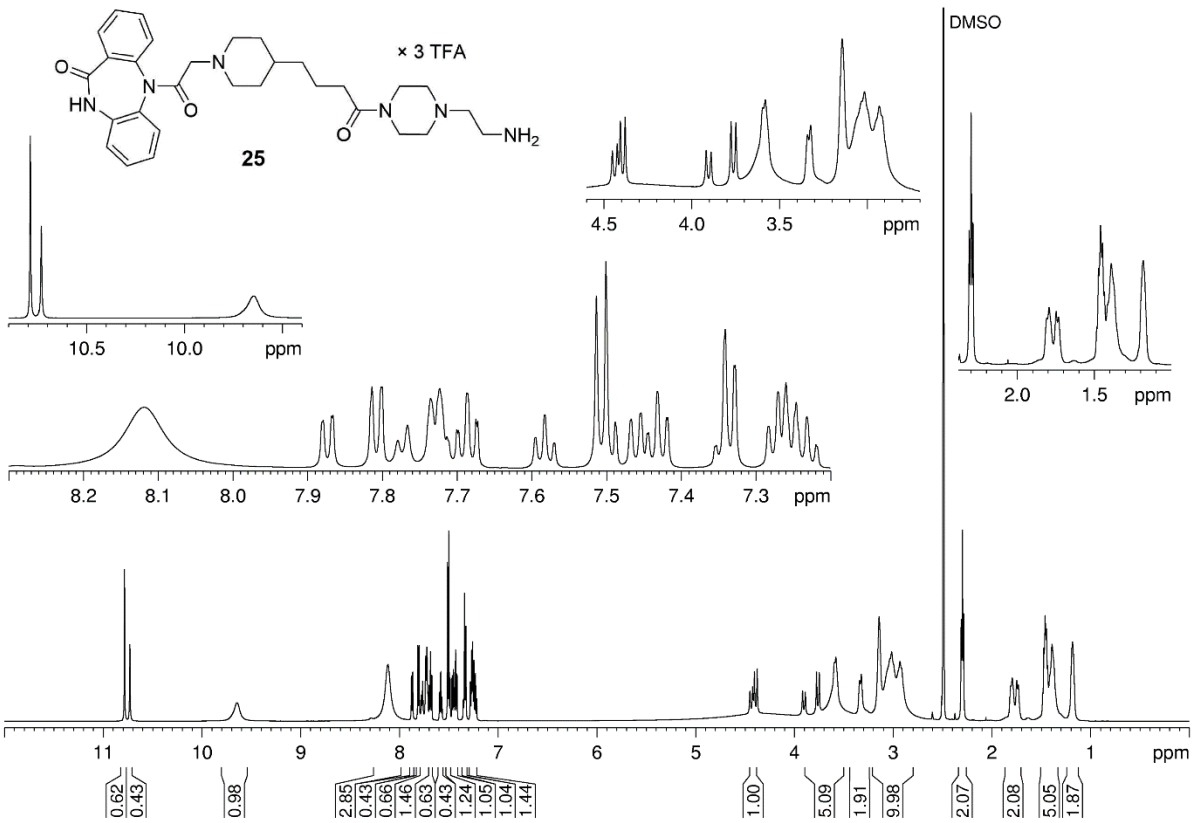
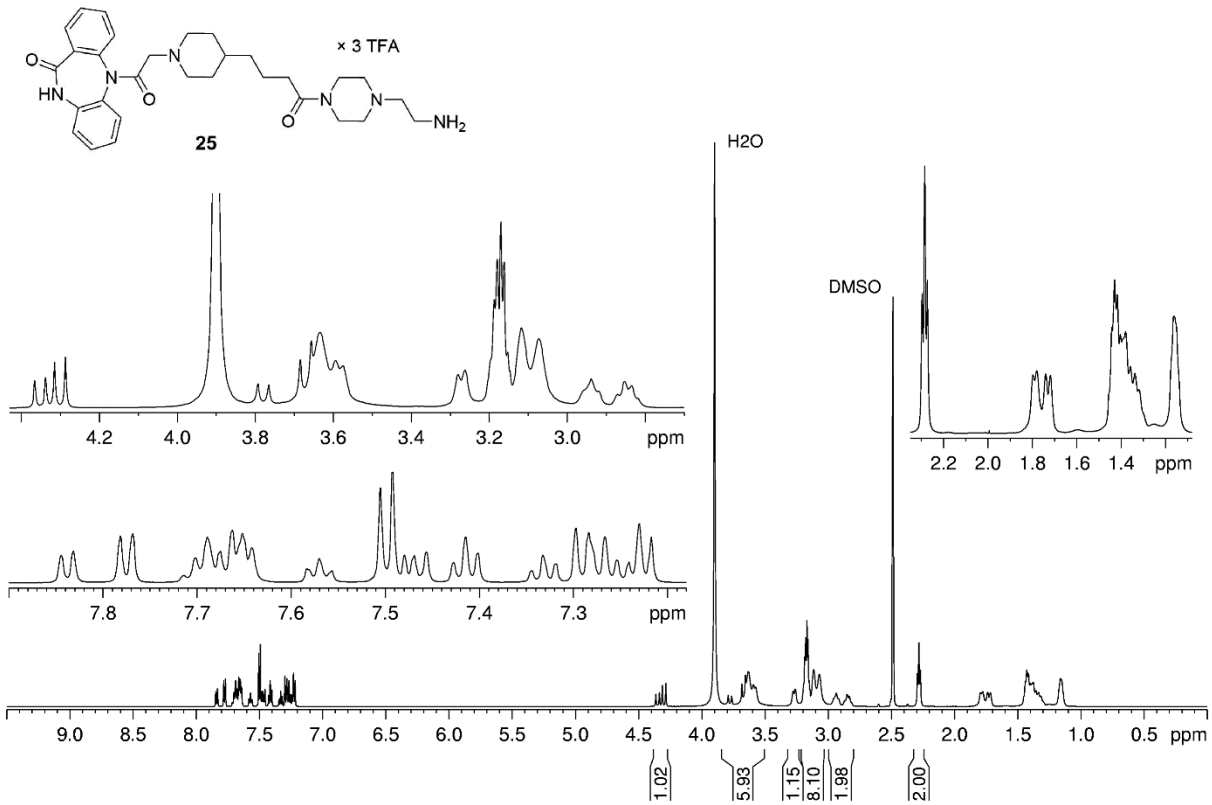


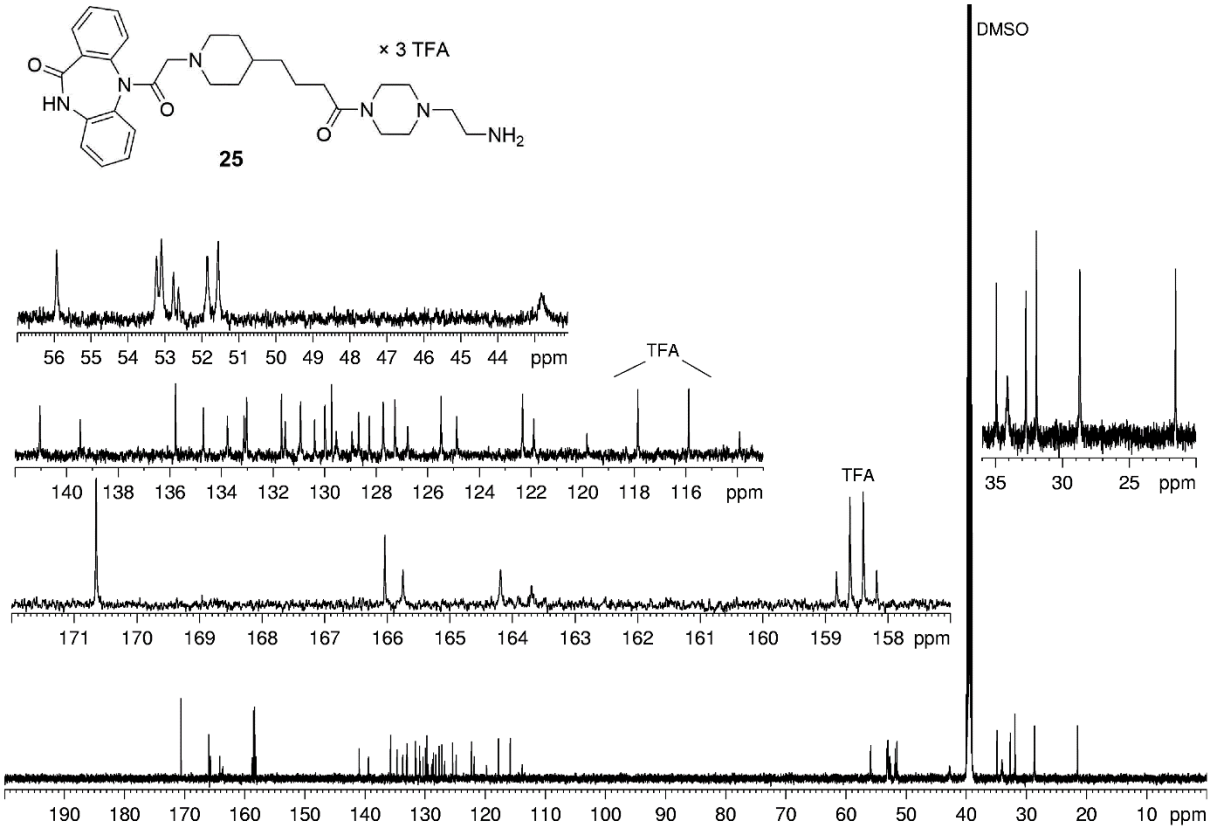
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **24**



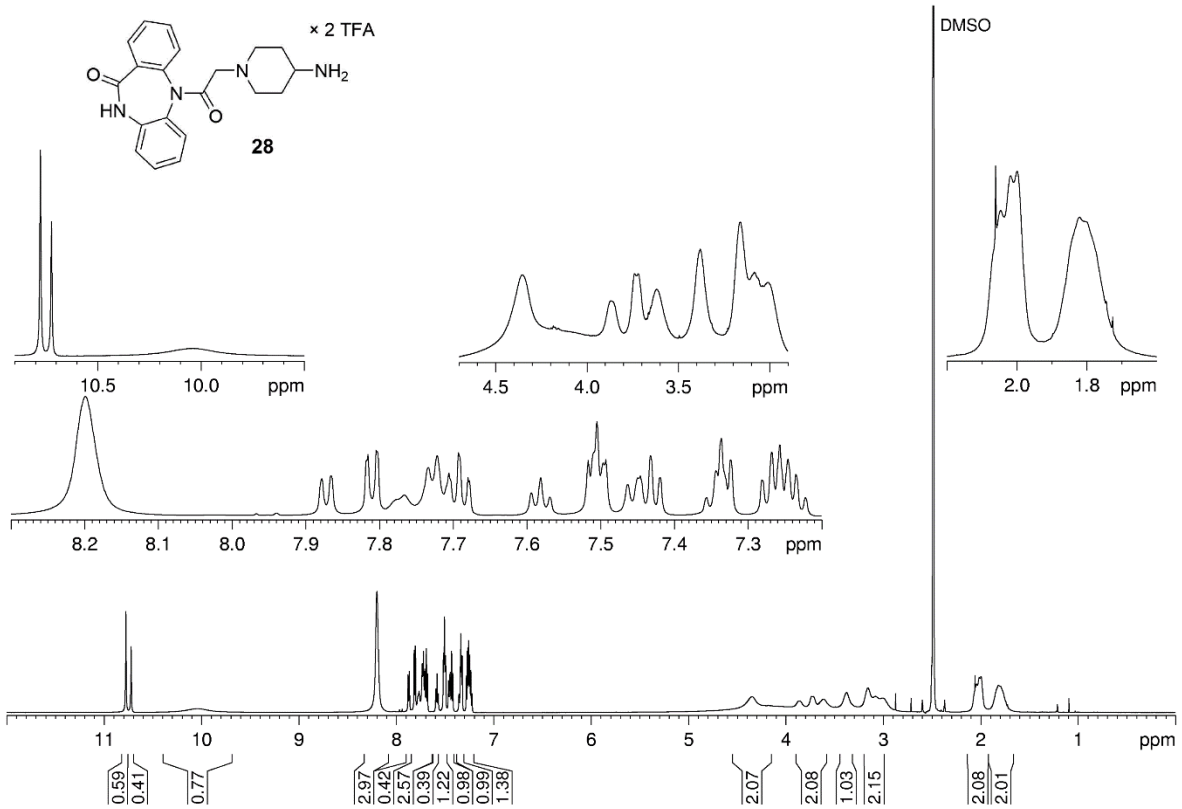
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **25**



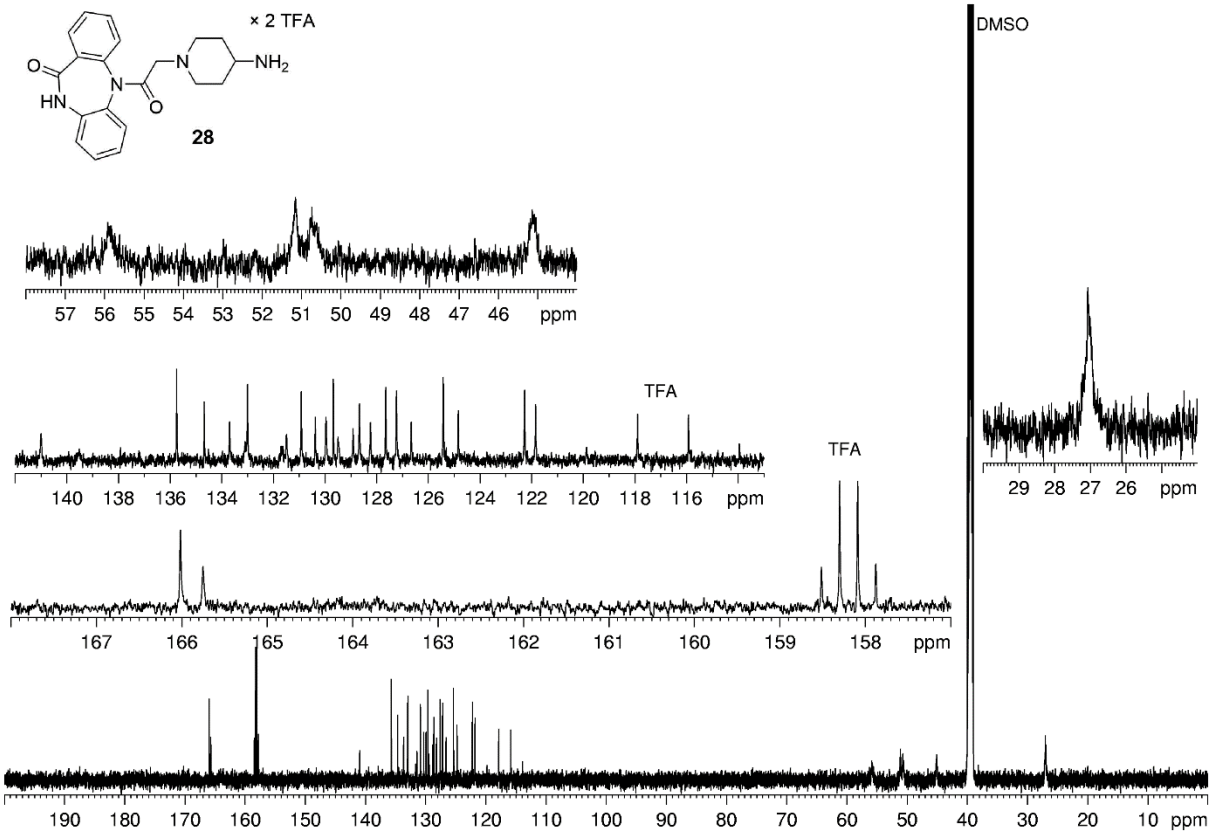
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **25**



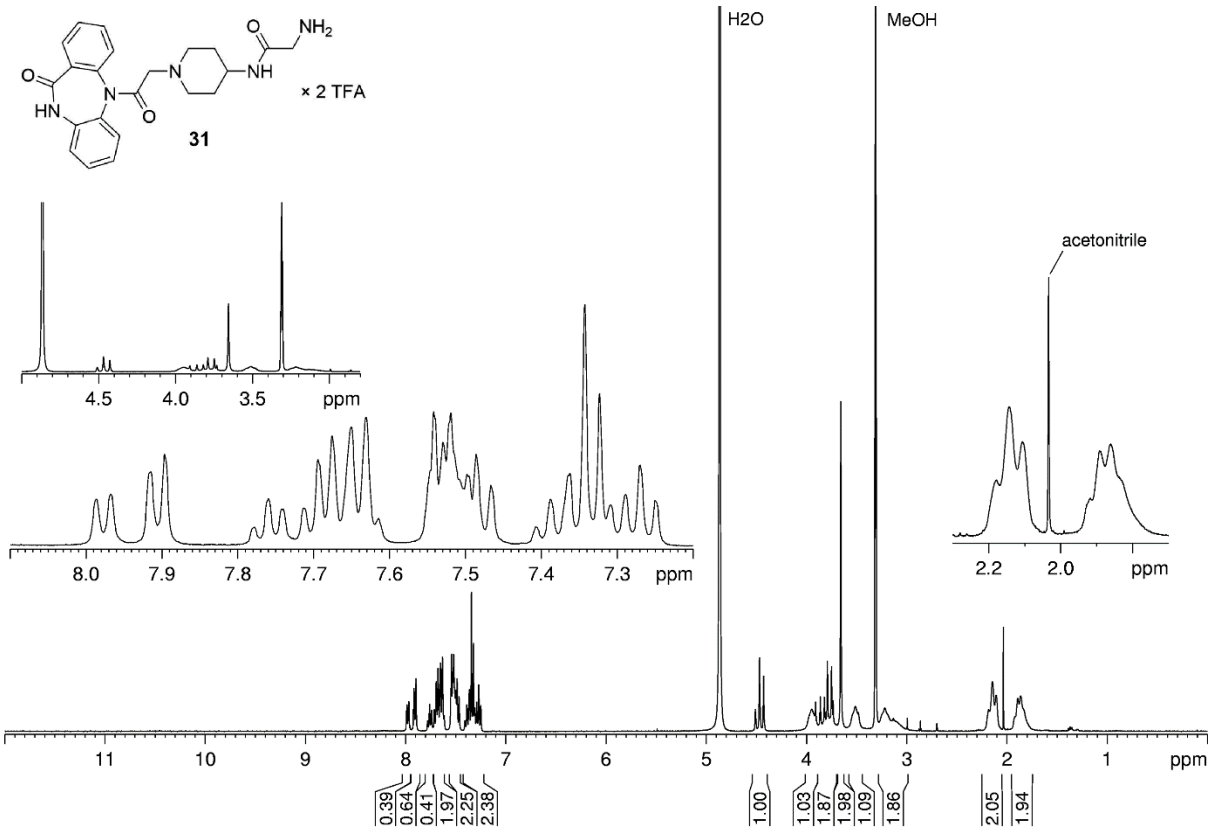
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **25**



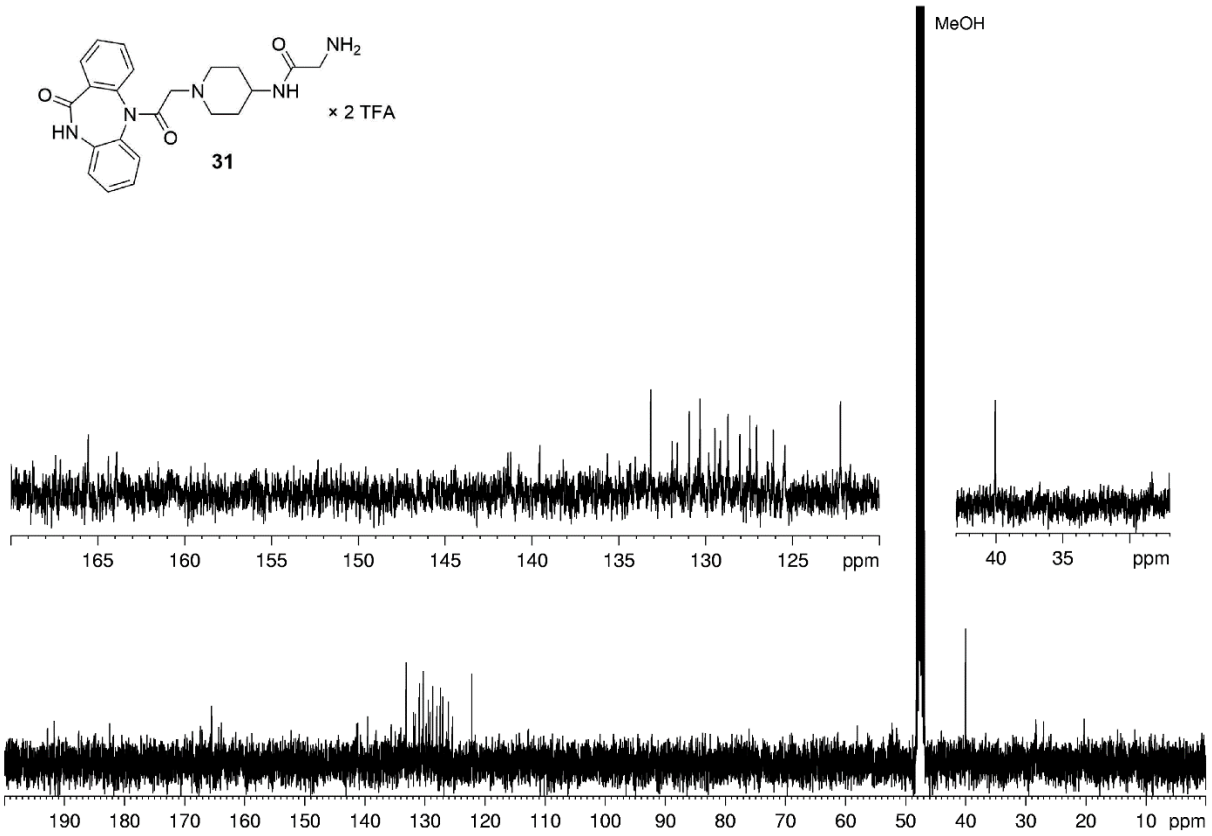
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **28**



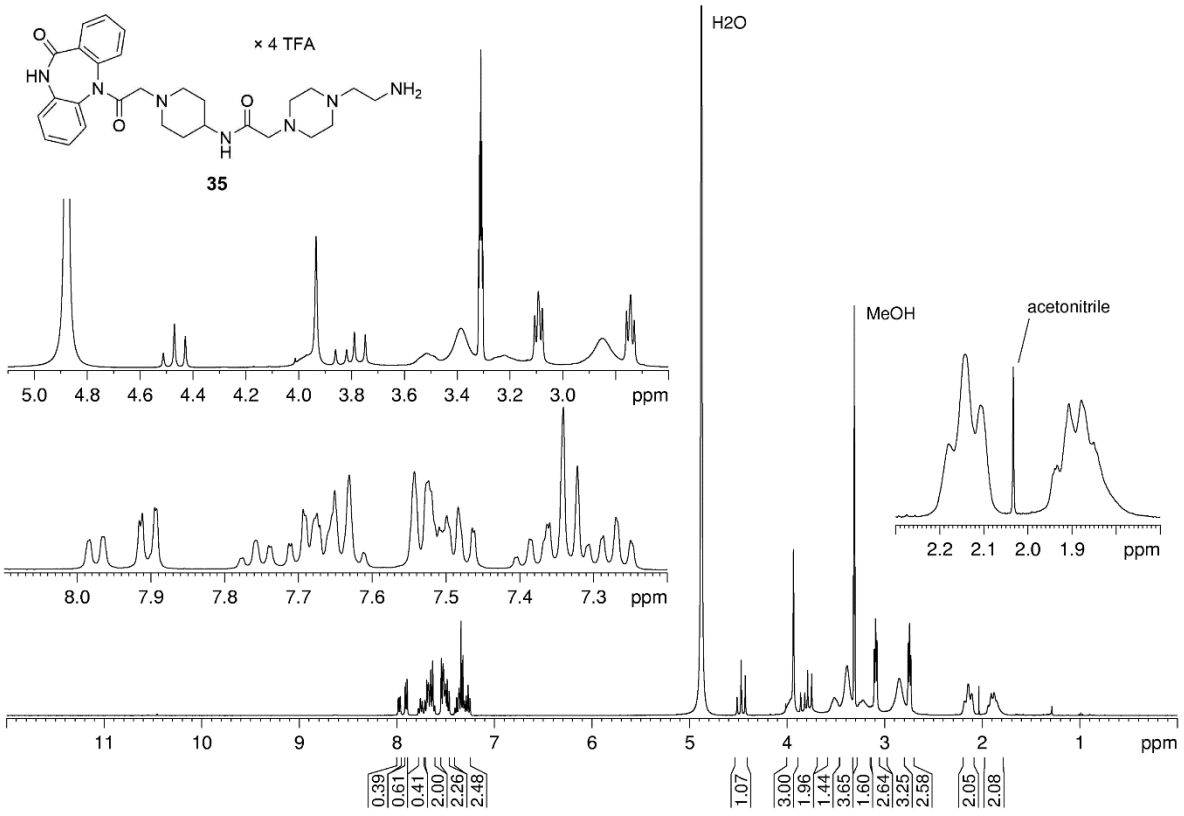
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **28**



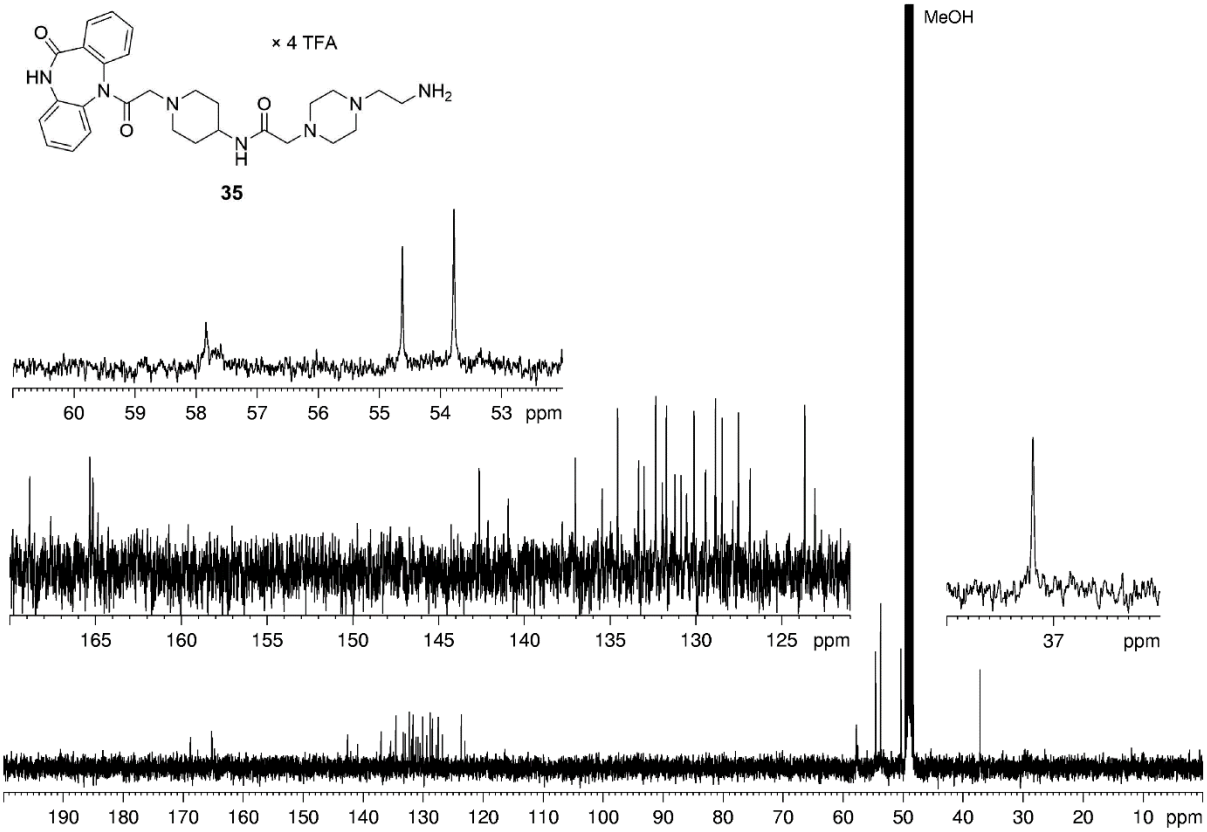
^{1}H-NMR spectrum (400 MHz, MeOH-d_4) of compound **31**



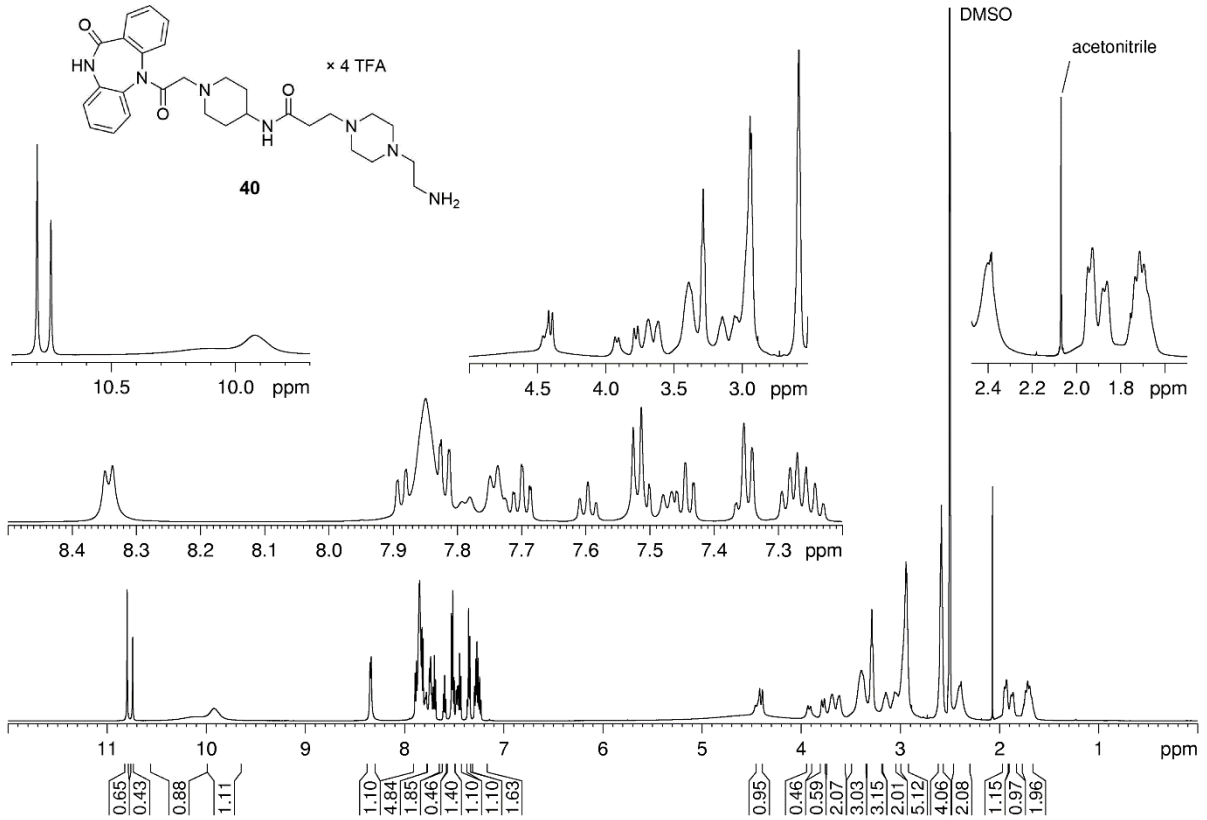
^{13}C-NMR spectrum (100 MHz, MeOH-d_4) of compound **31**



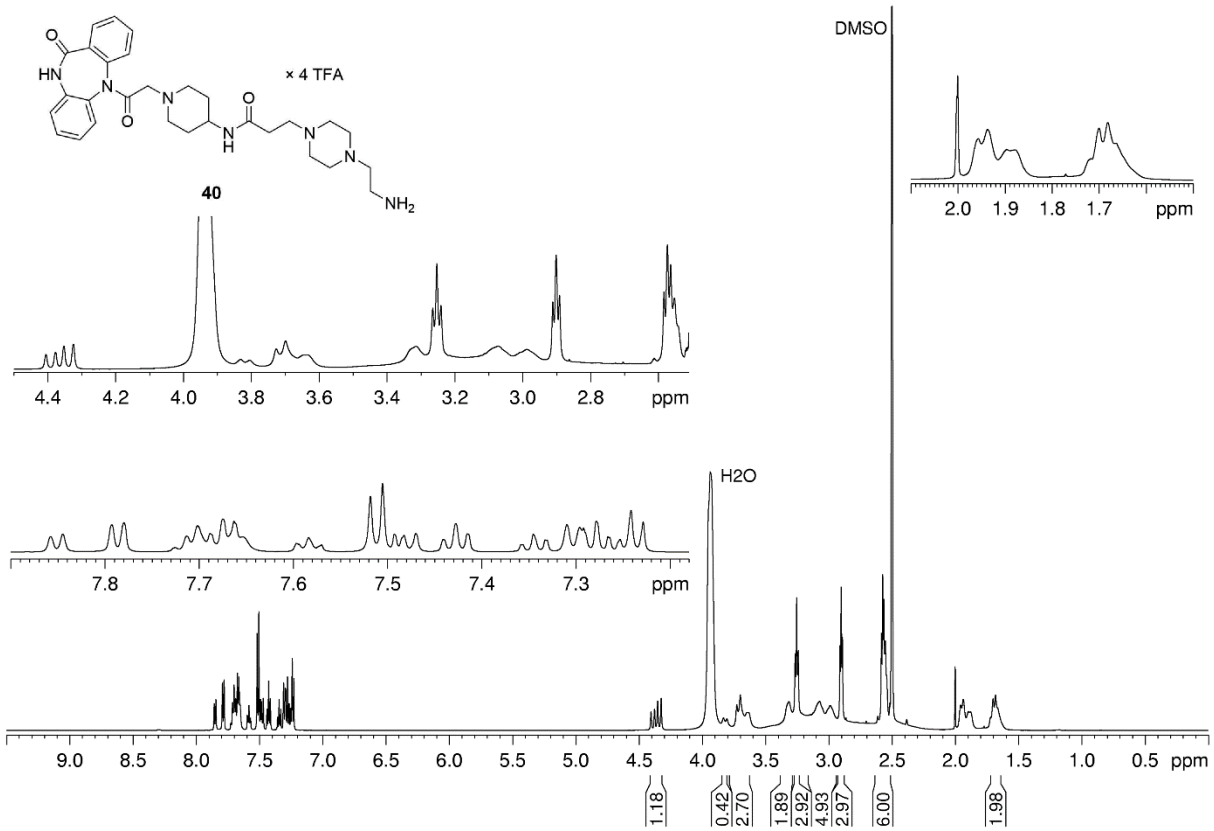
^{1}H-NMR spectrum (400 MHz, MeOH-d_4) of compound **35**



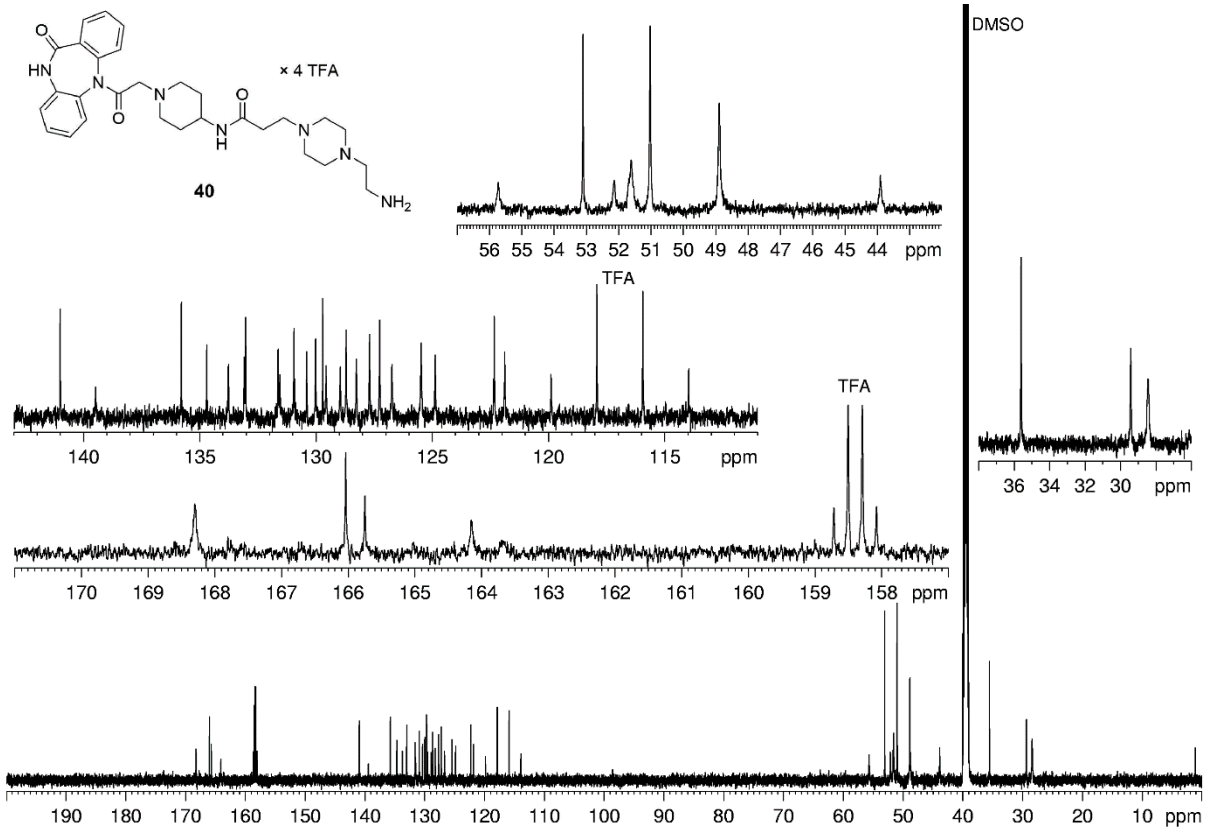
^{13}C-NMR spectrum (100 MHz, MeOH-d_4) of compound **35**



^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **40**



^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **40**



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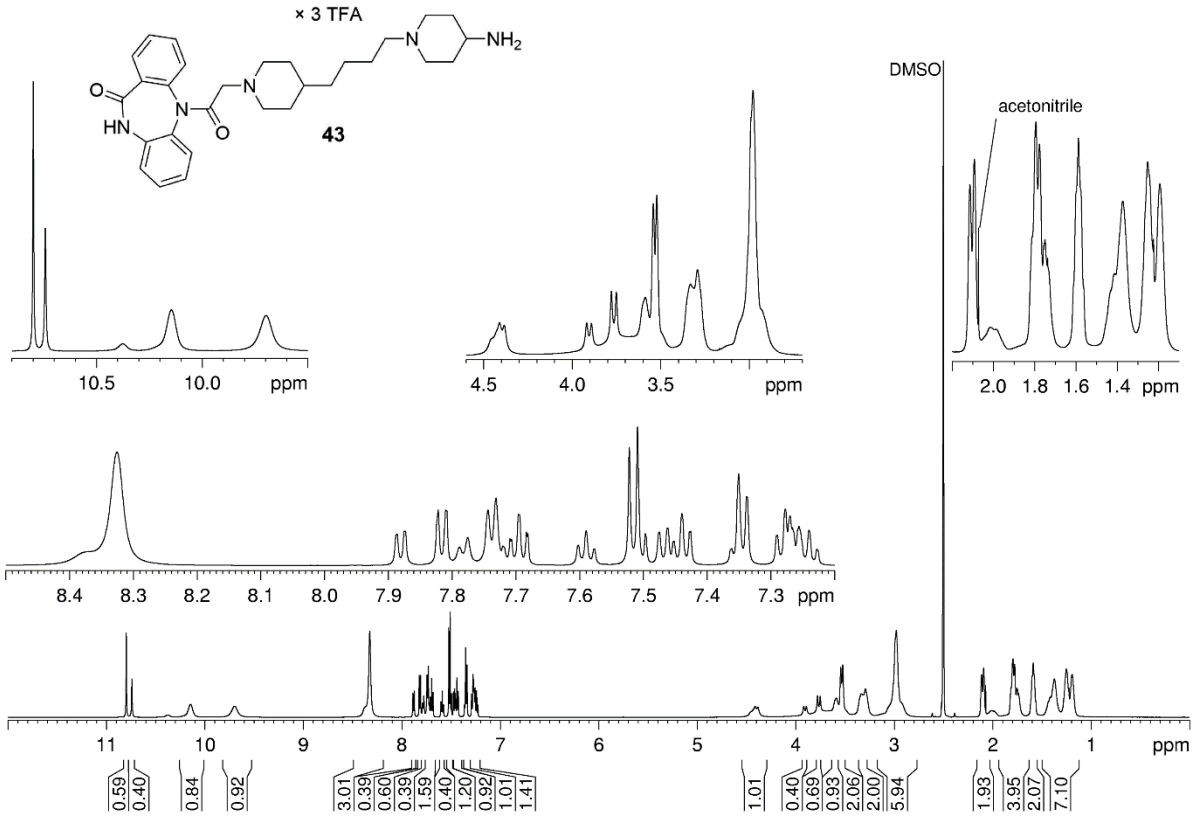

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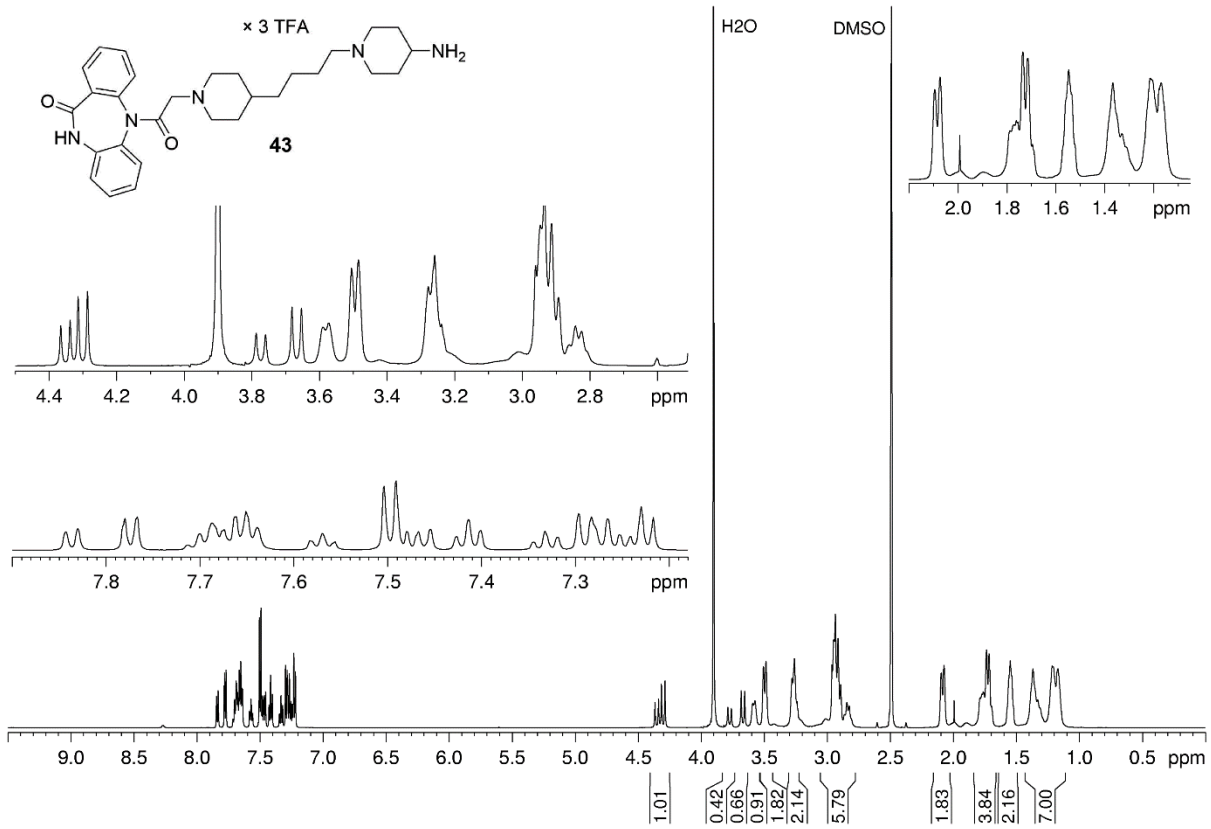

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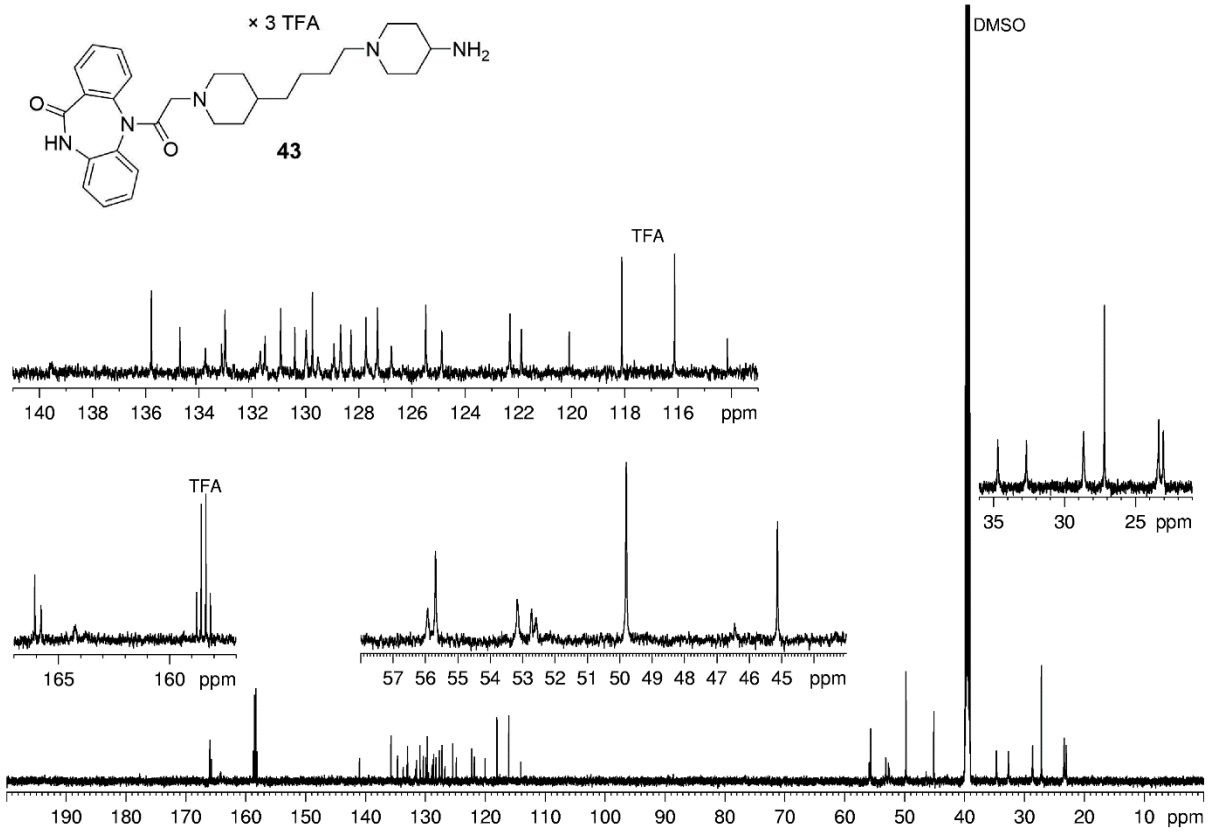
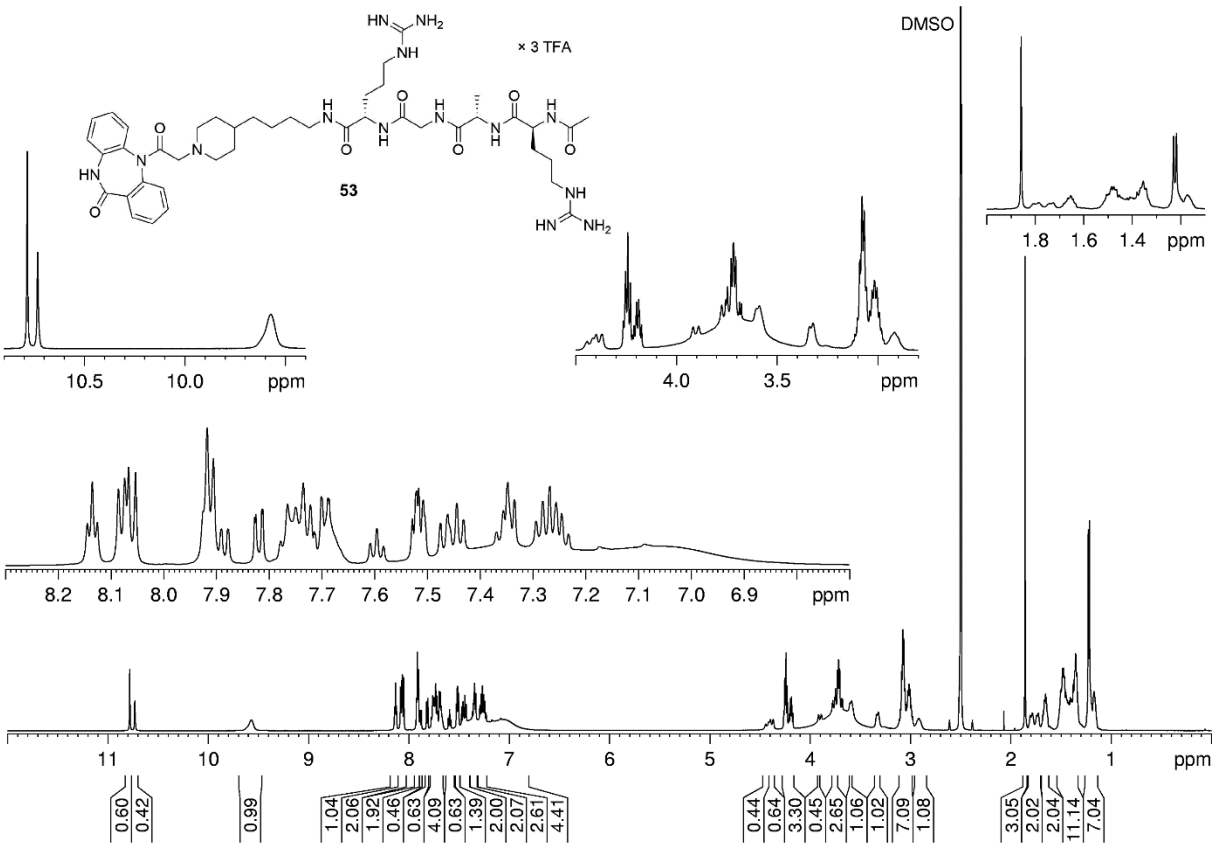


^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **43**



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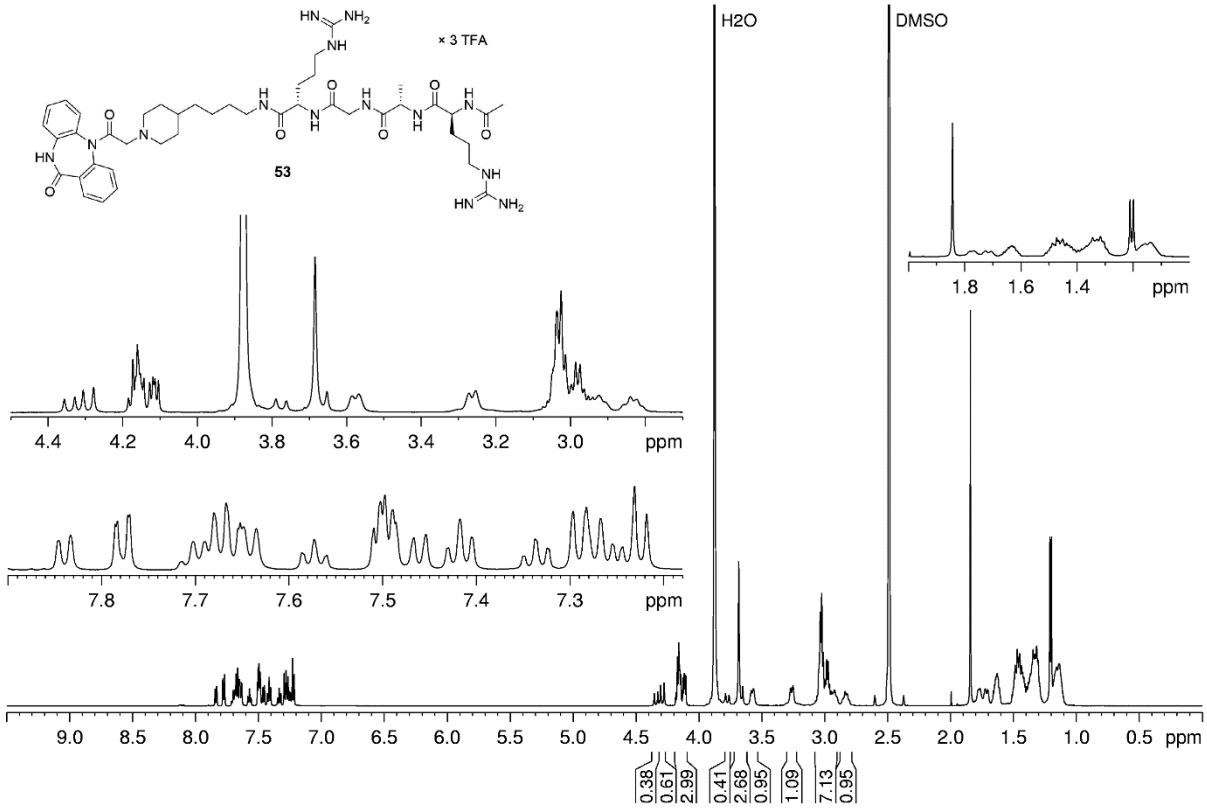

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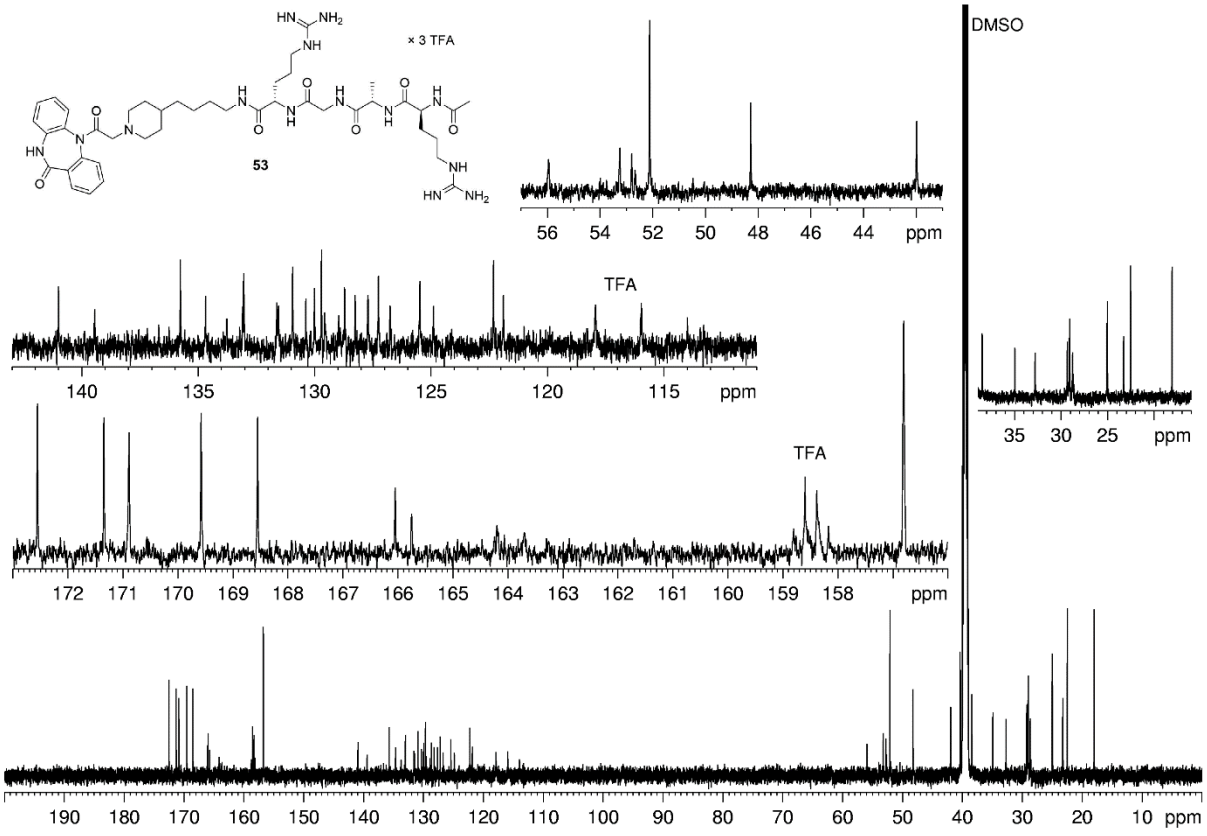

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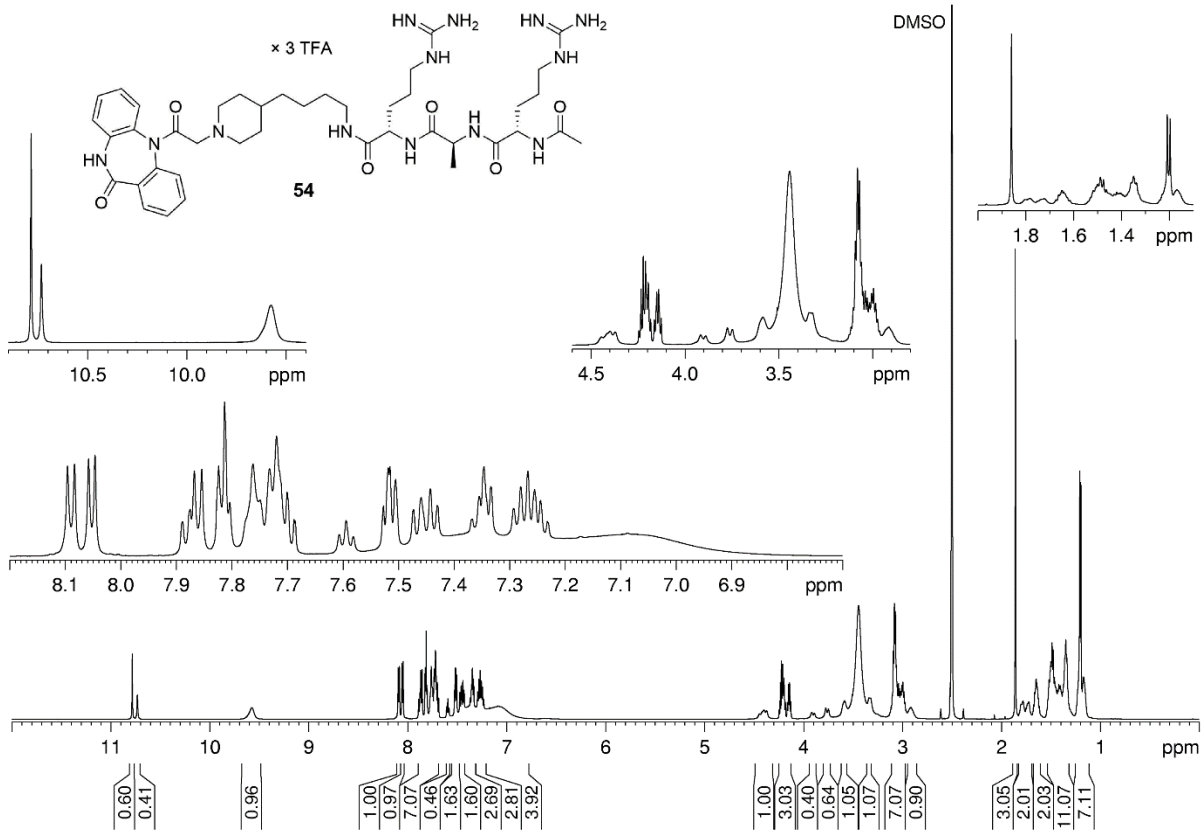

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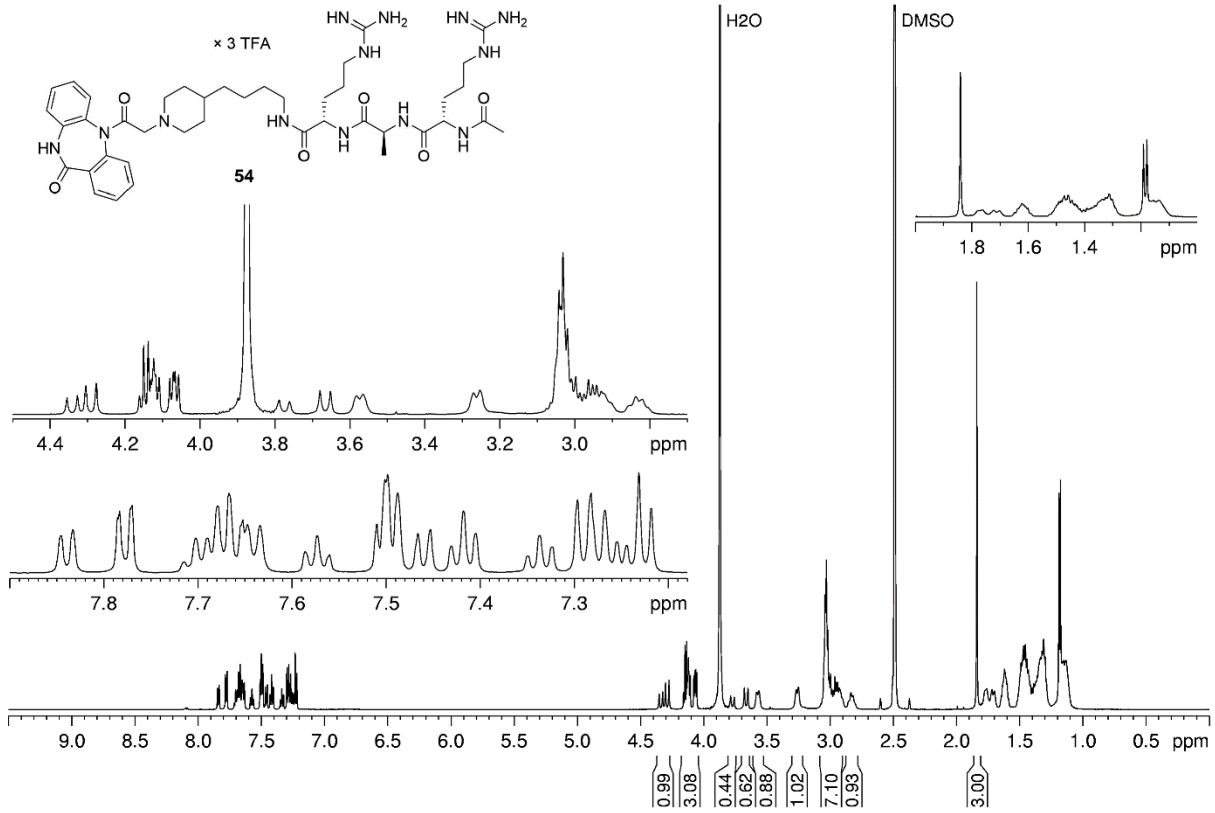

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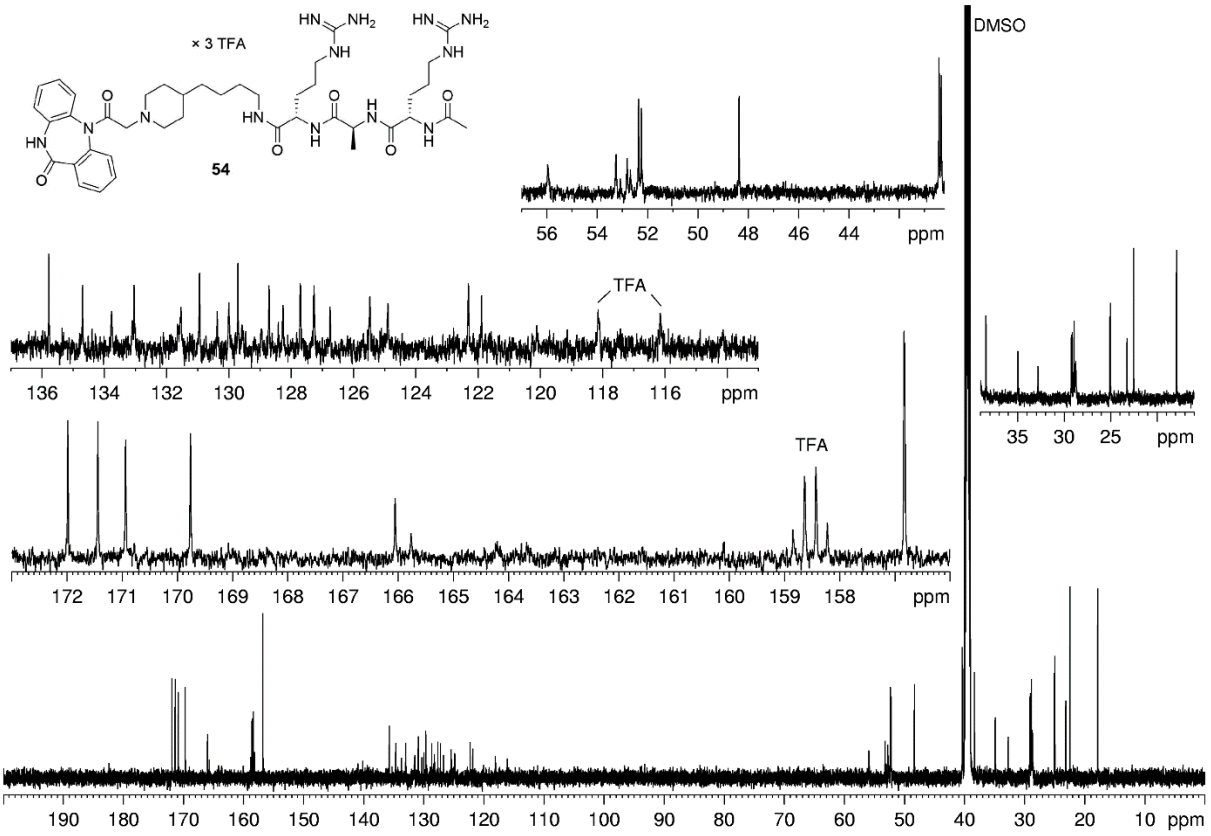
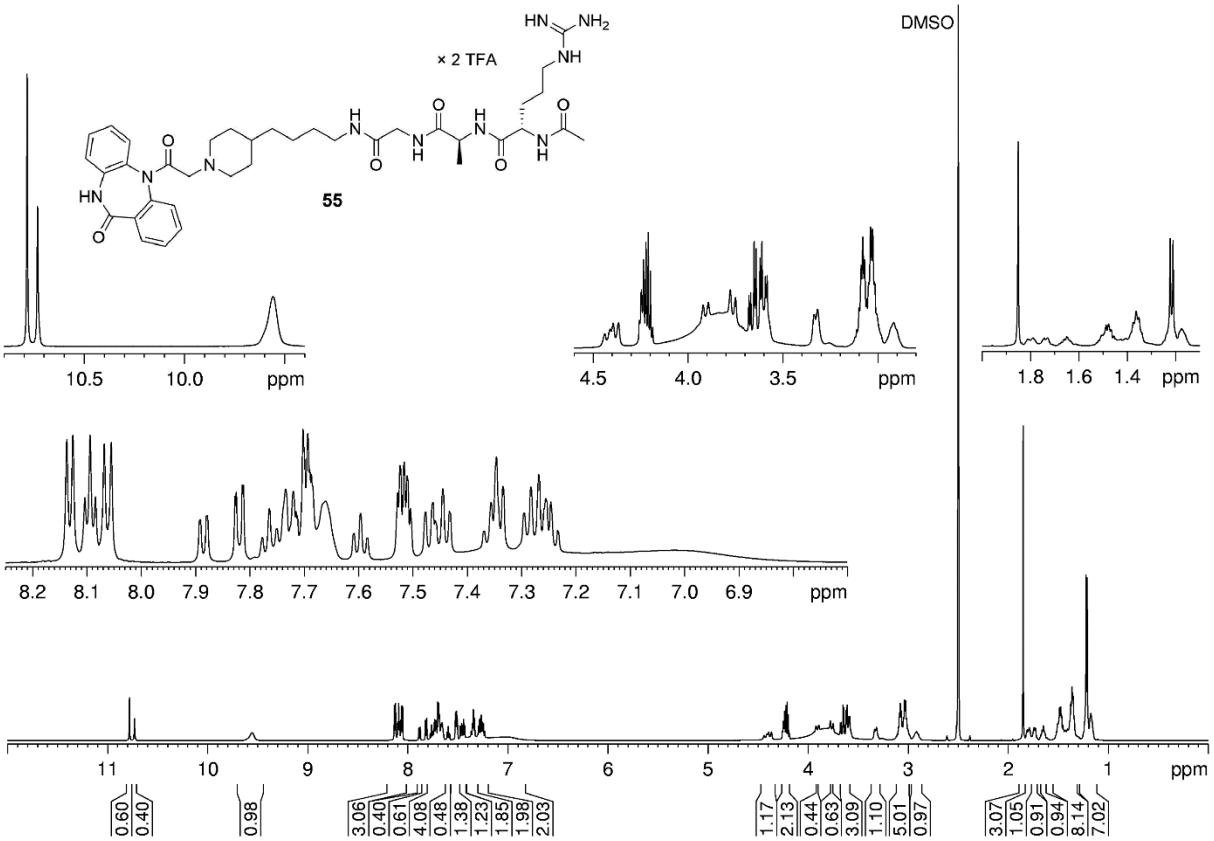
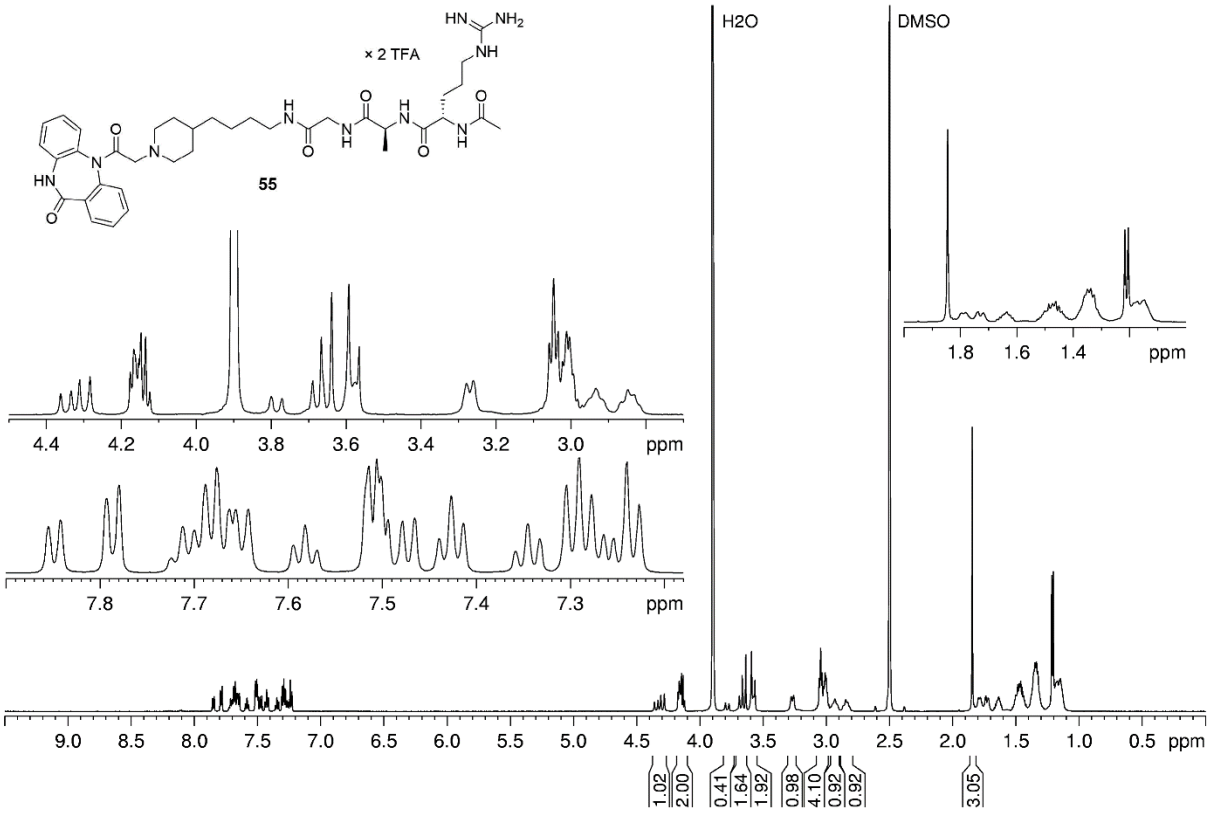


^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **54**



^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **55**



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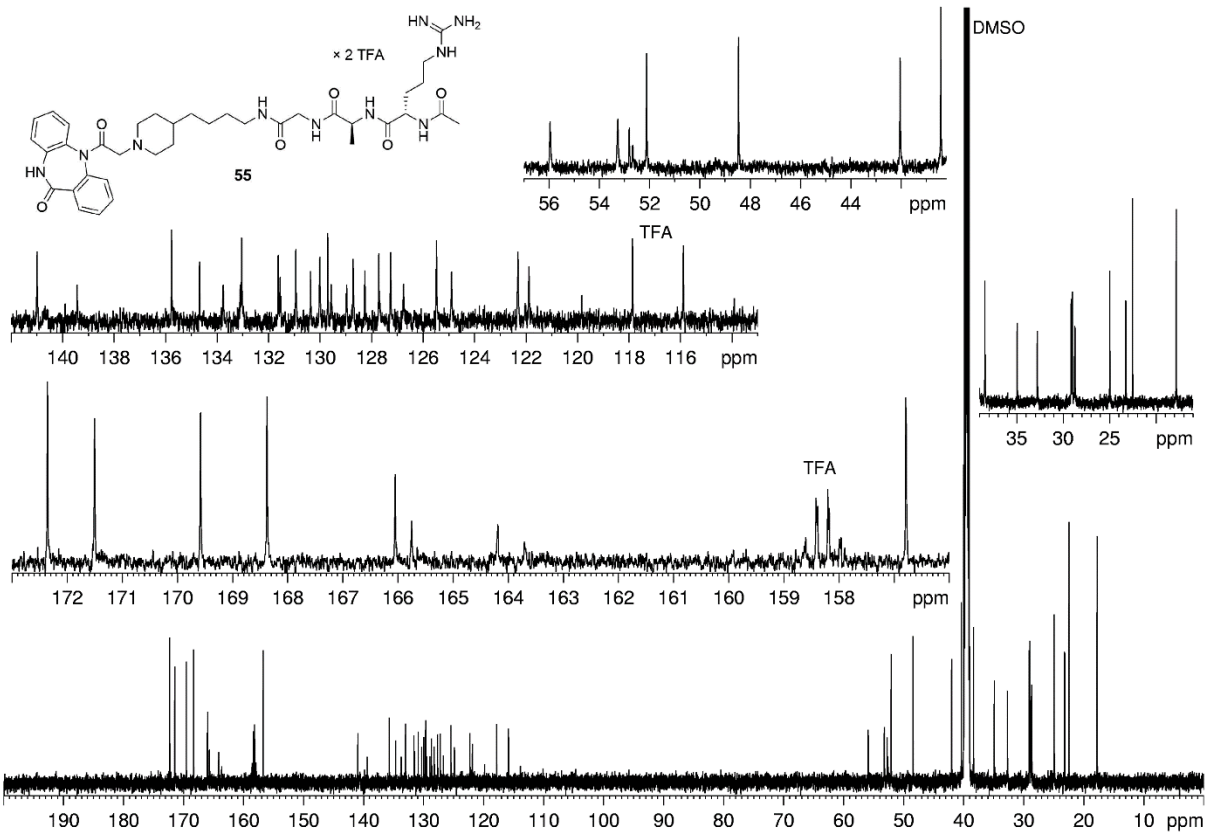
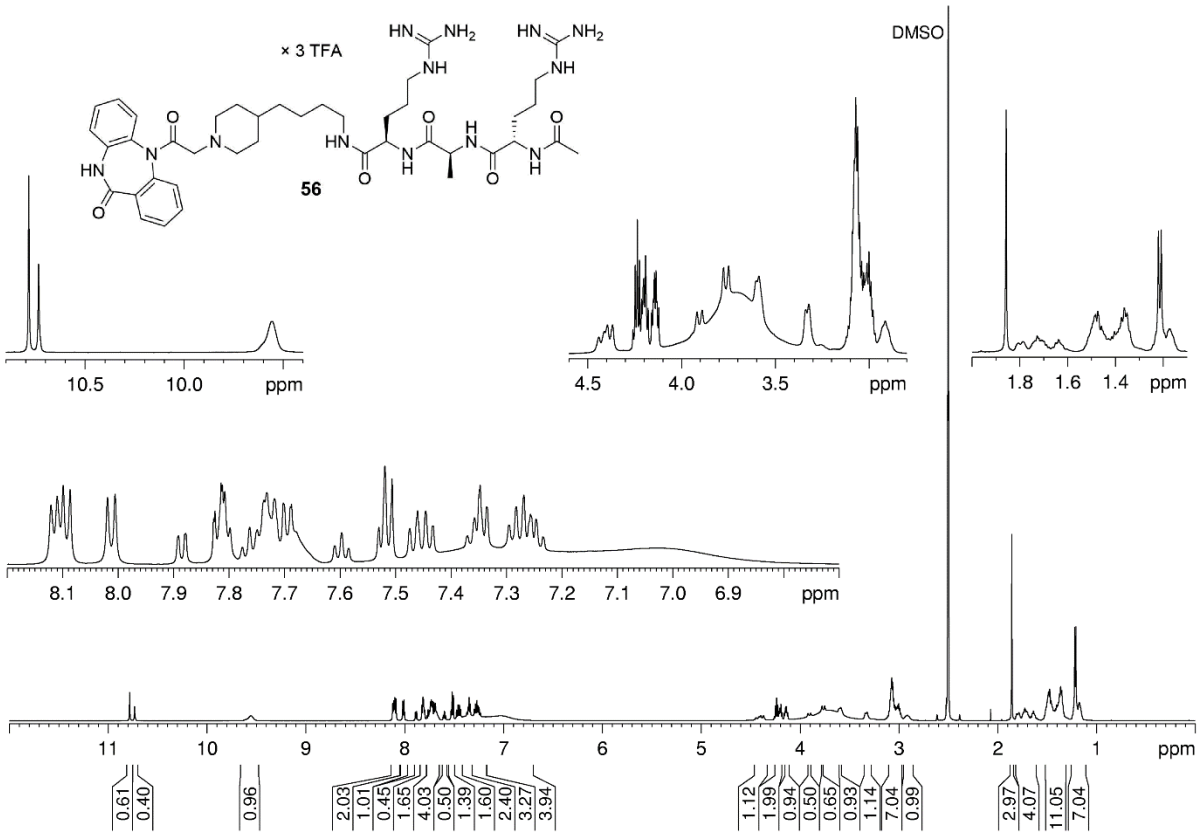
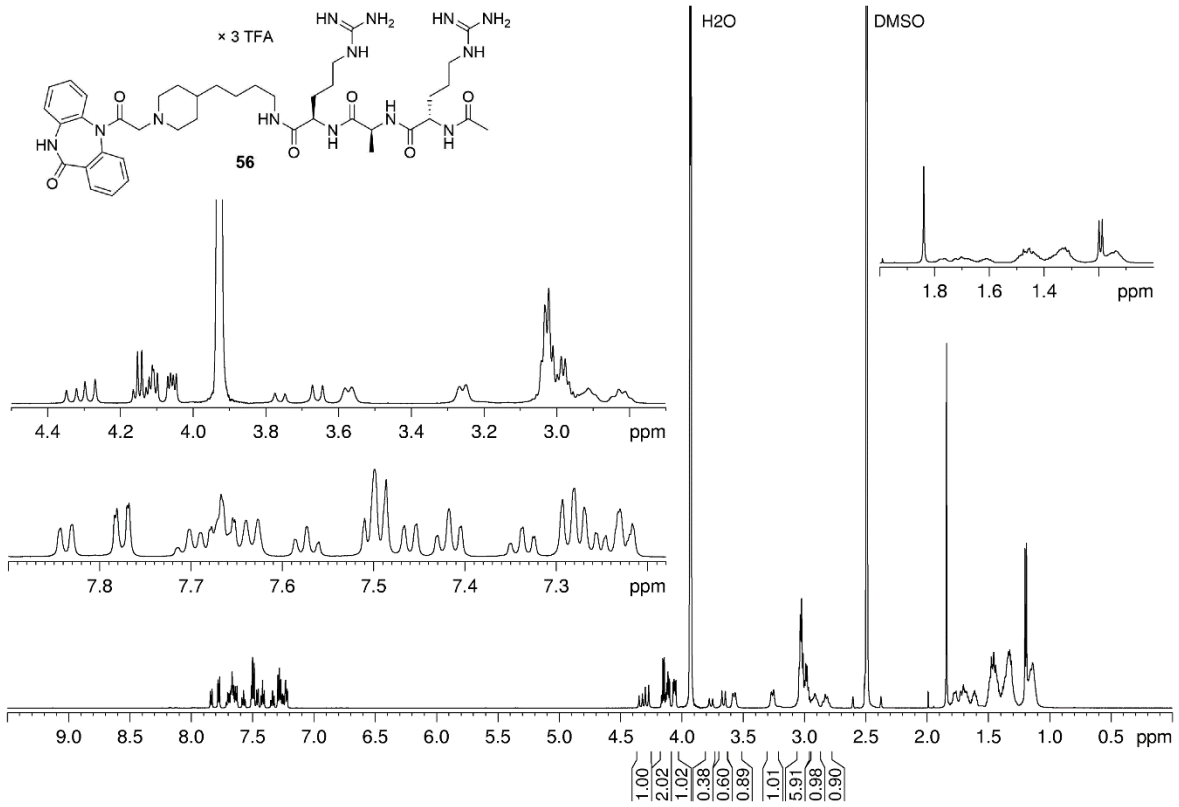


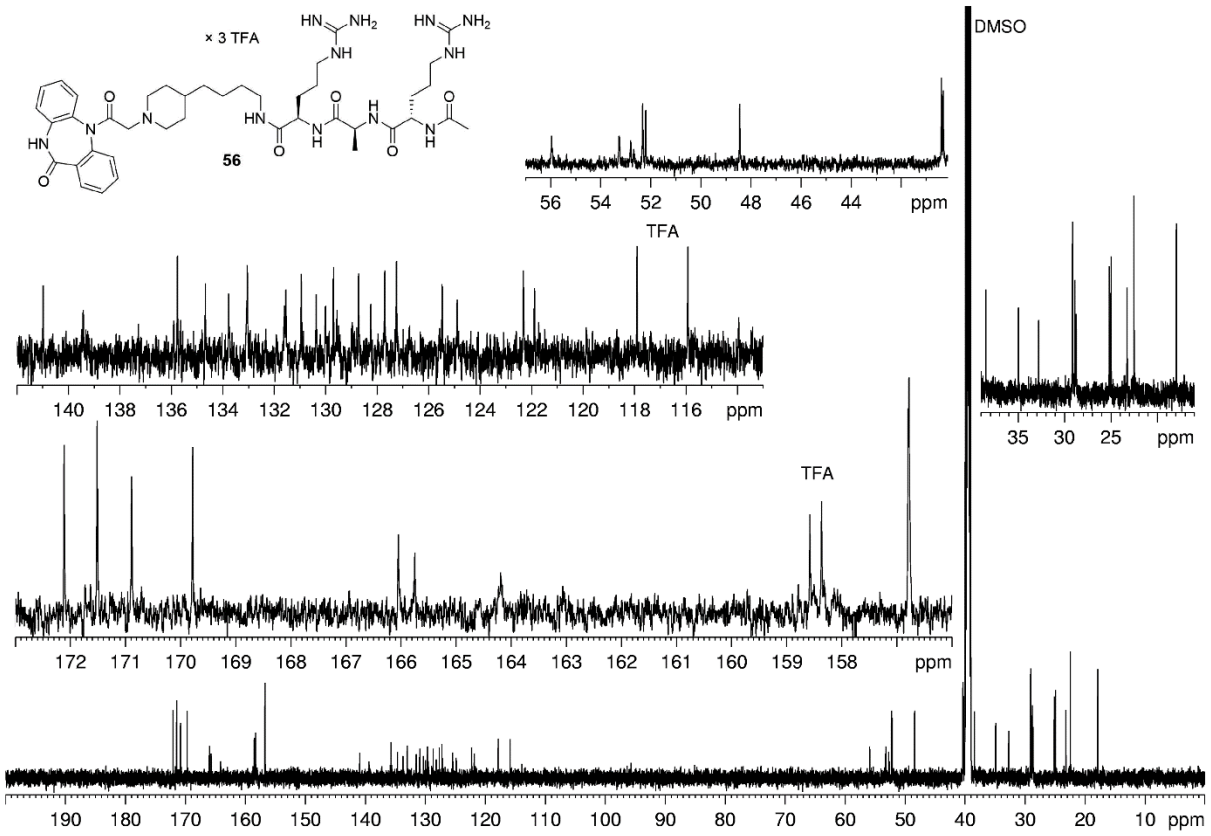
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **55**



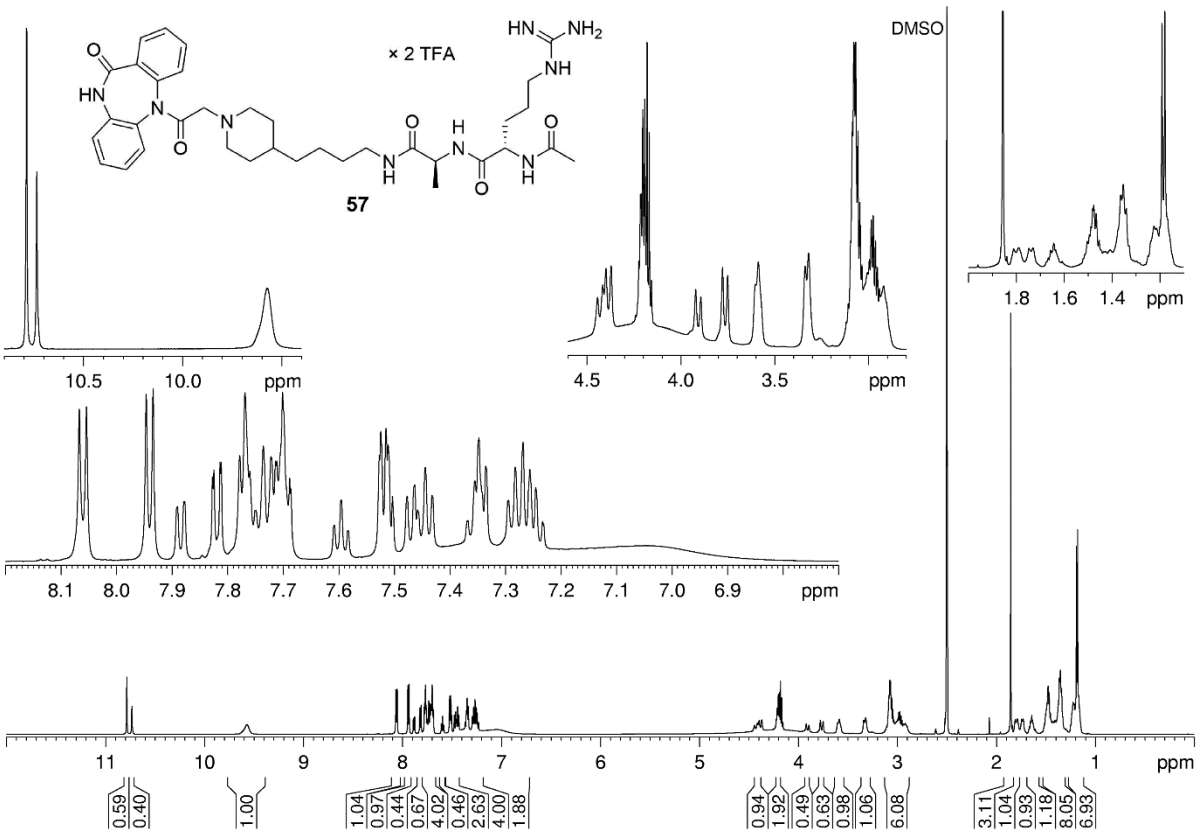
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **56**



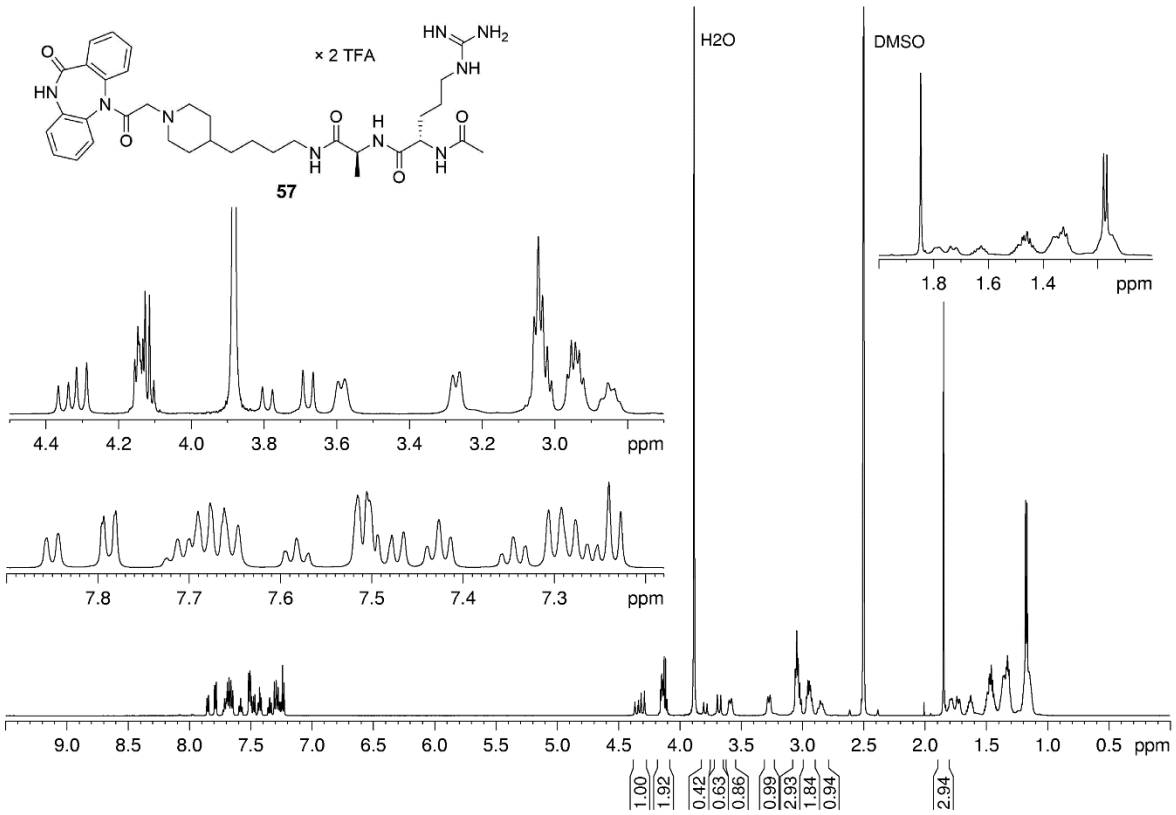
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **56**



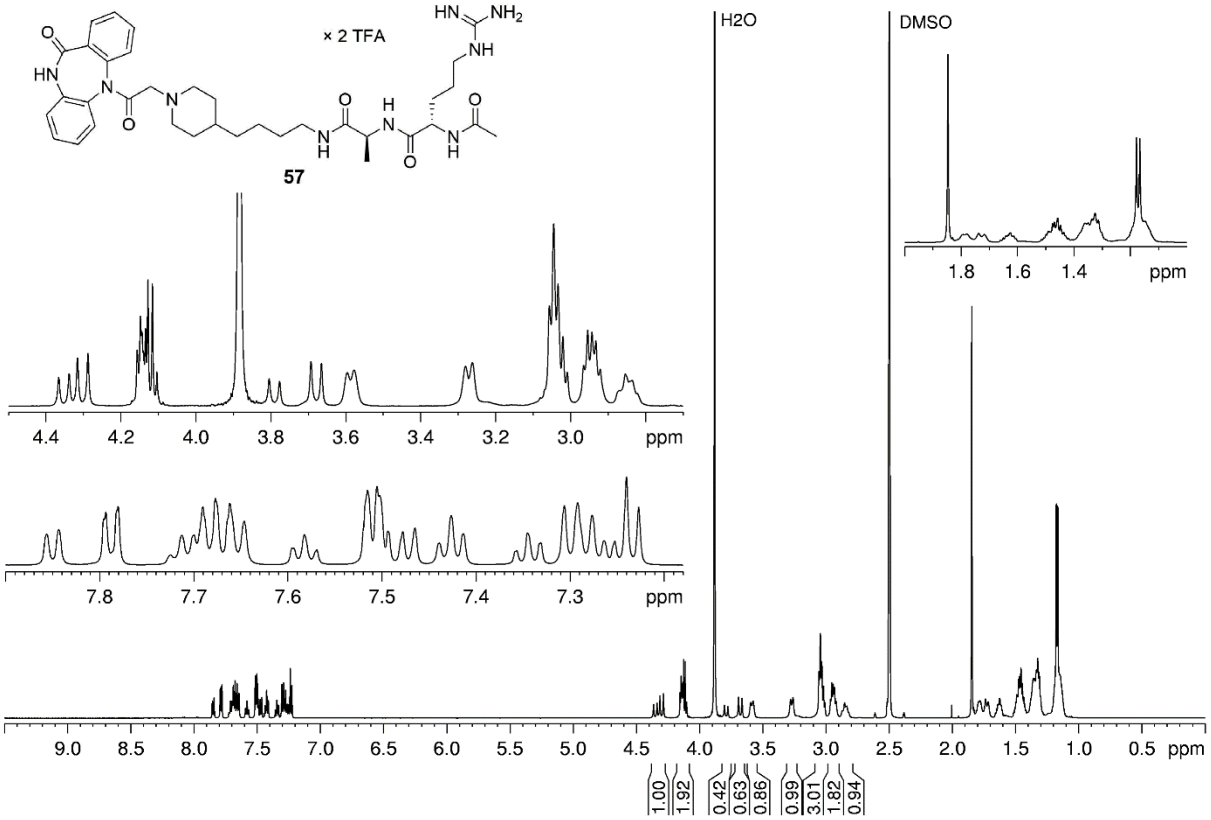
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **56**



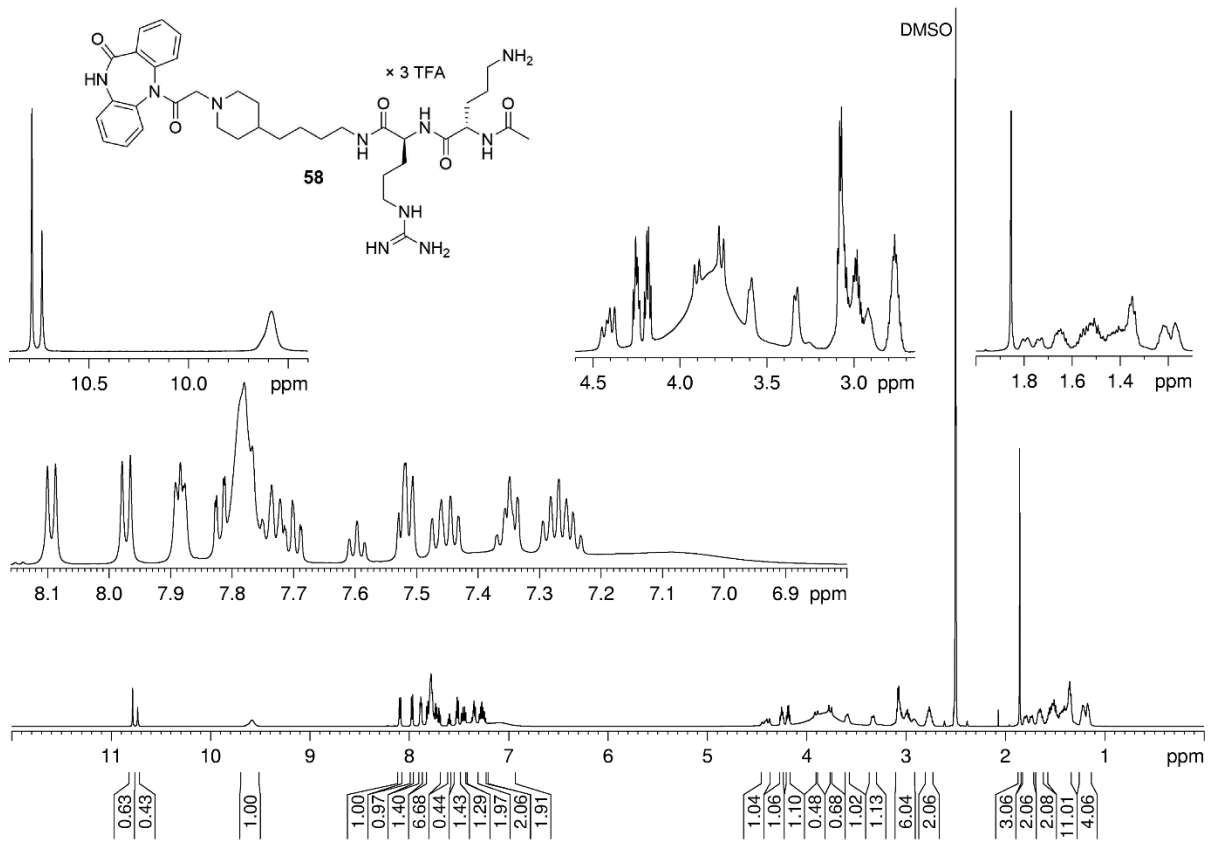
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **57**



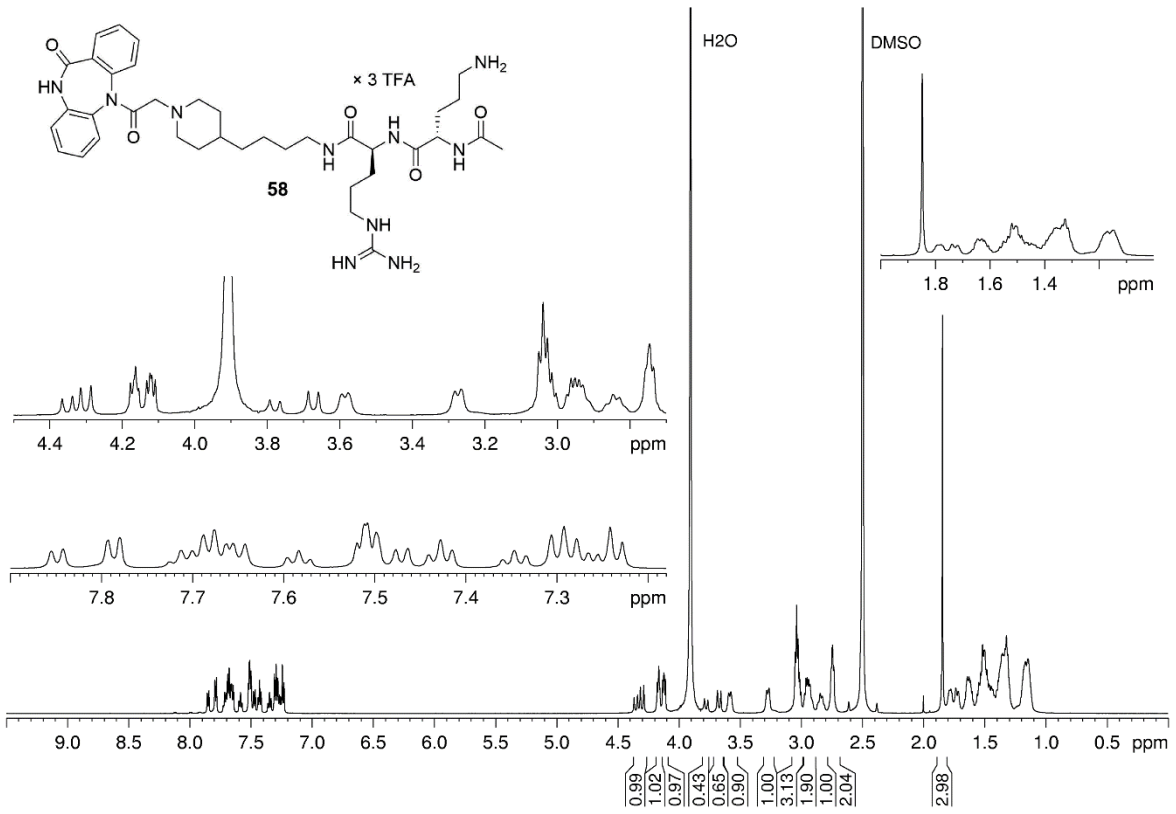
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **57**



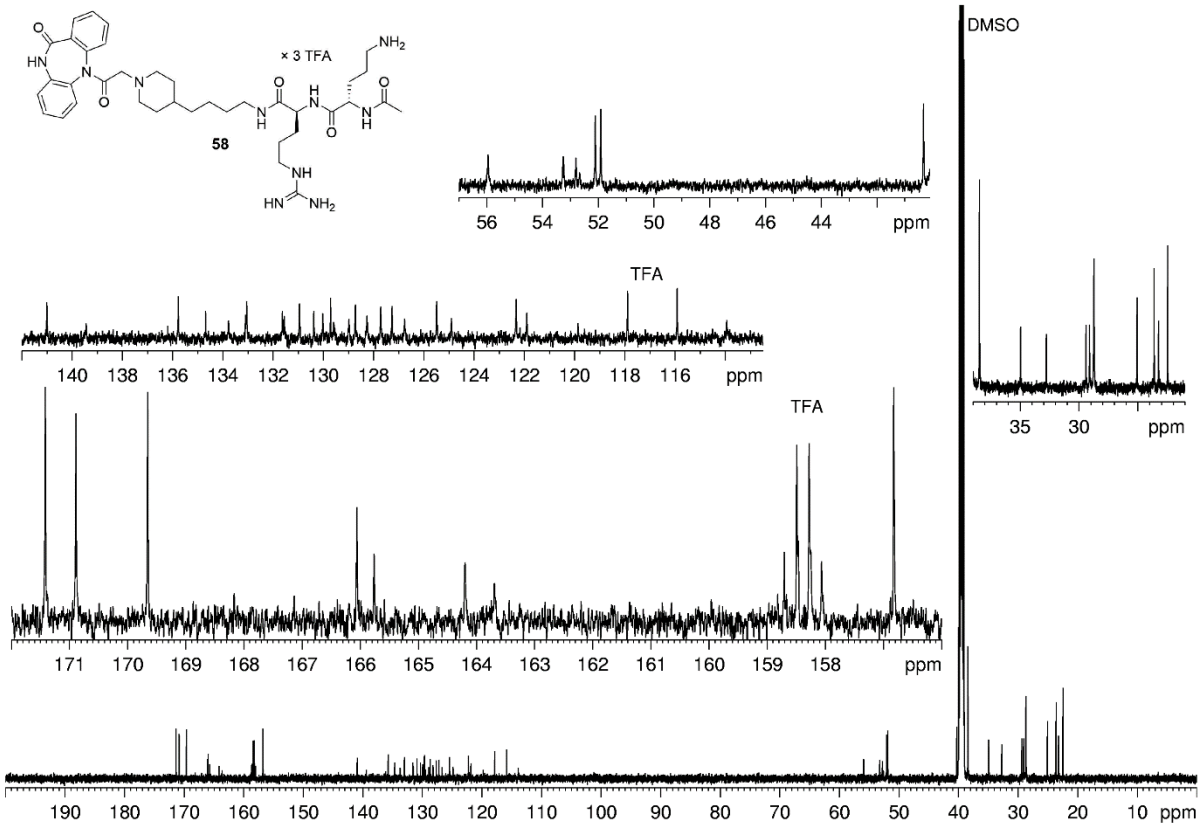
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **57**



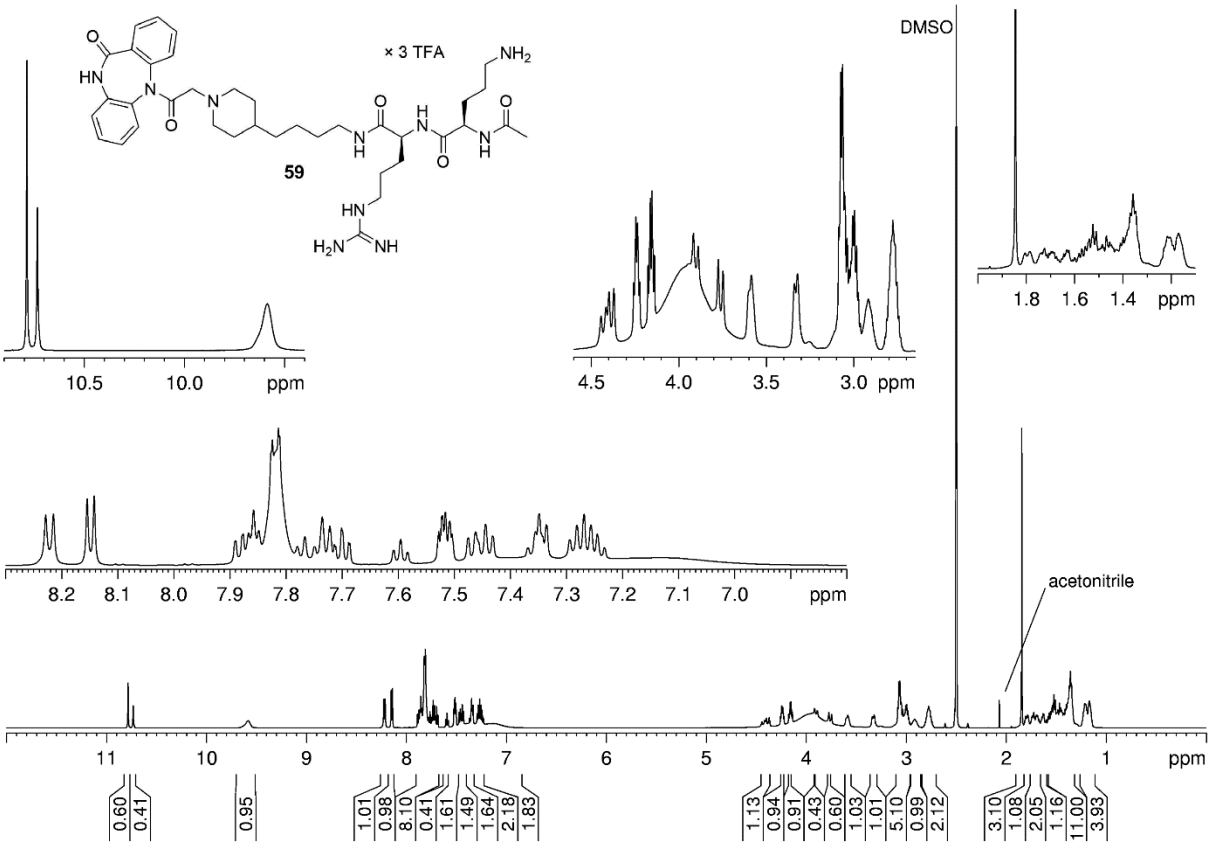
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **58**



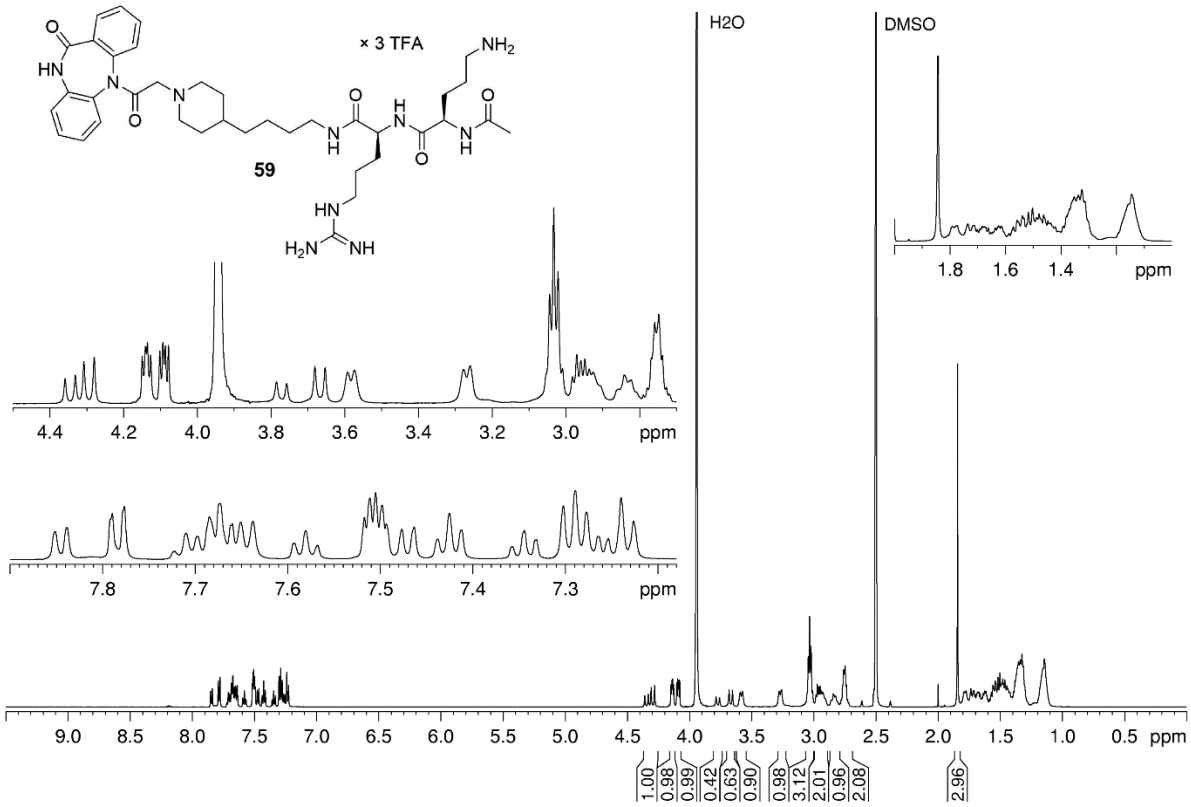
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **58**



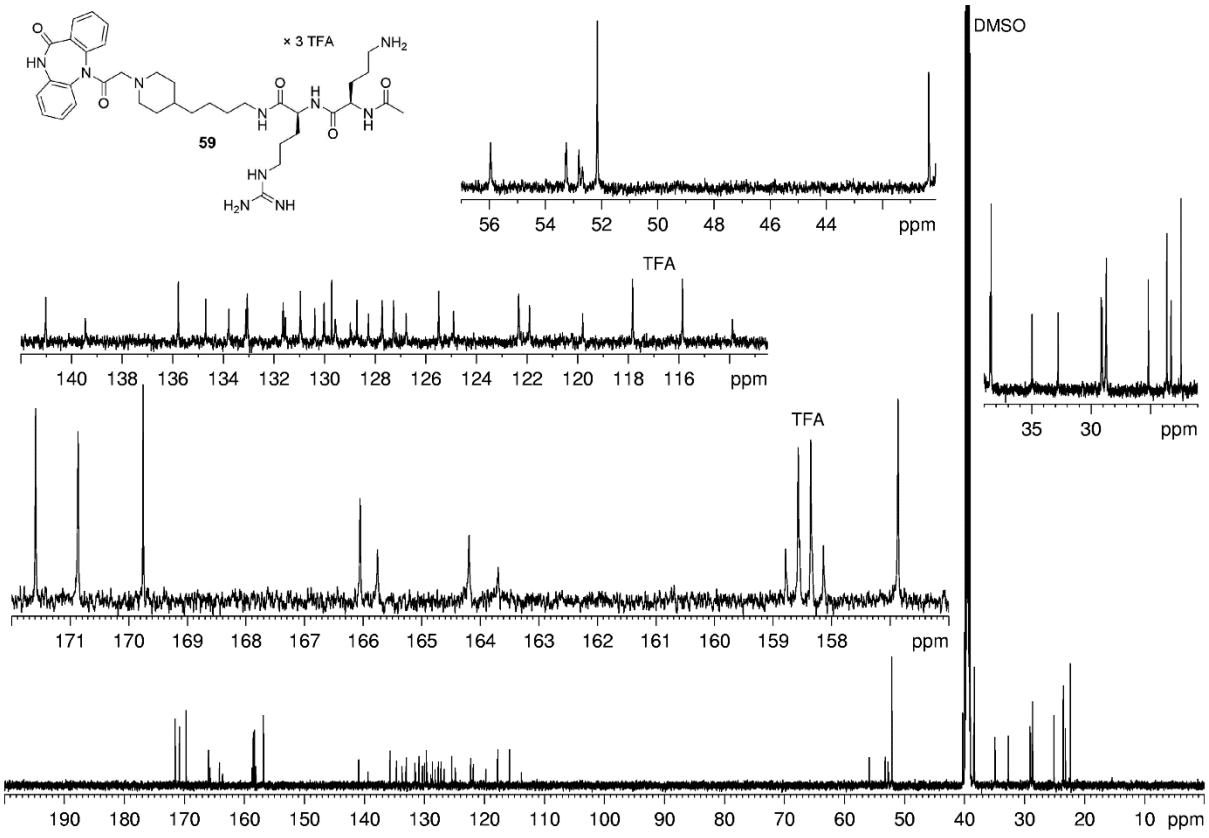
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **58**



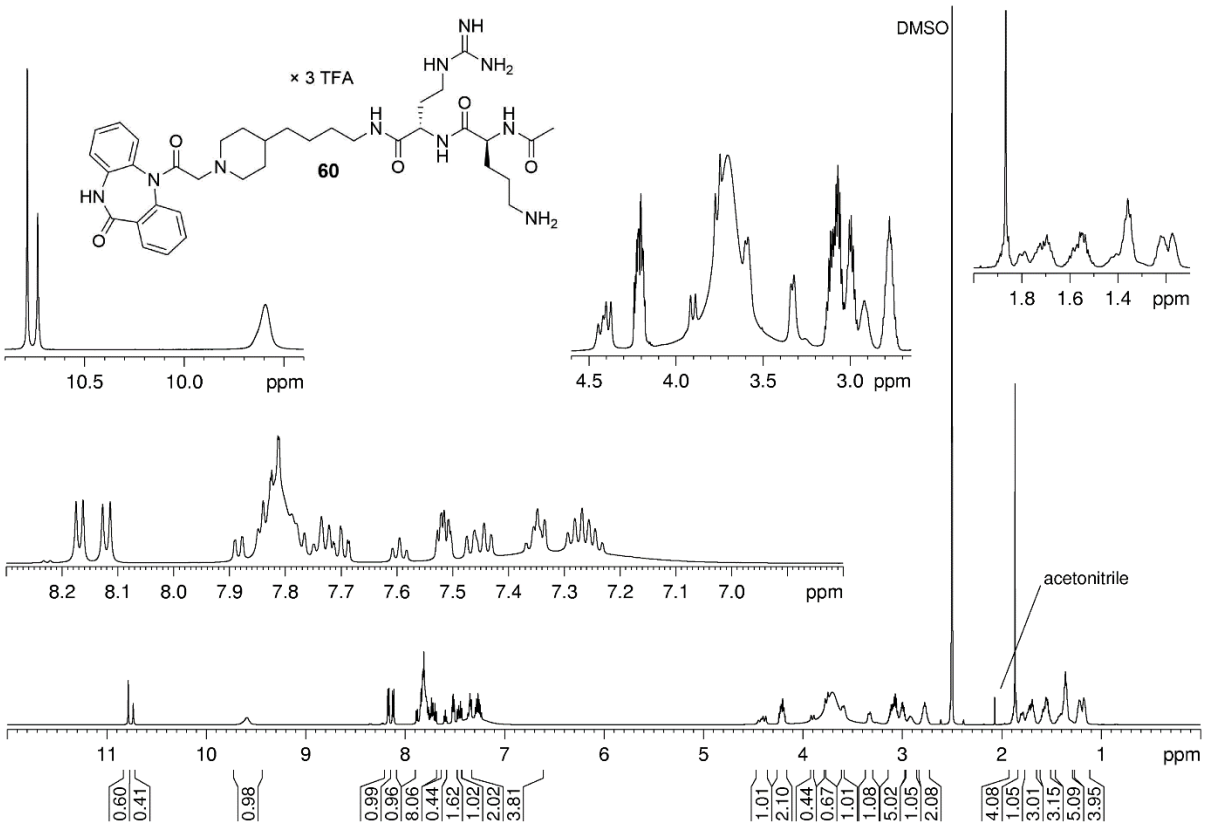
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **59**



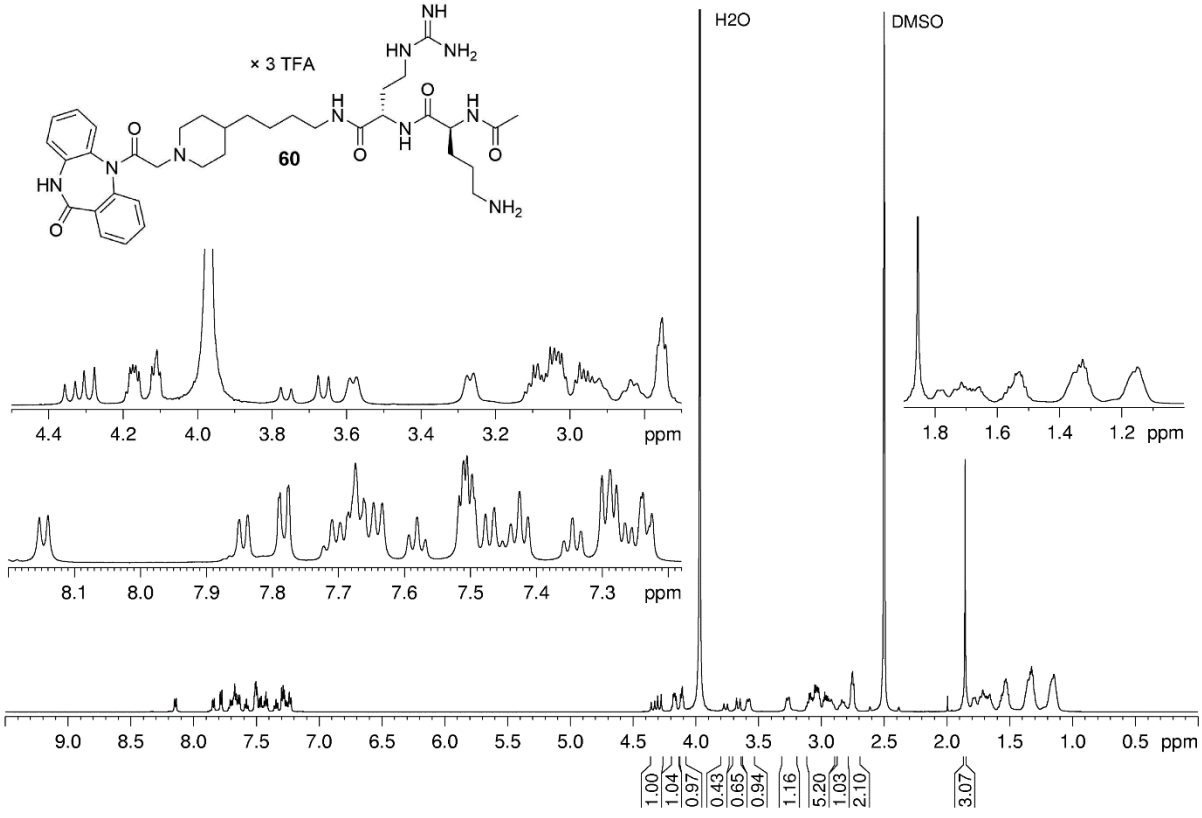
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **59**



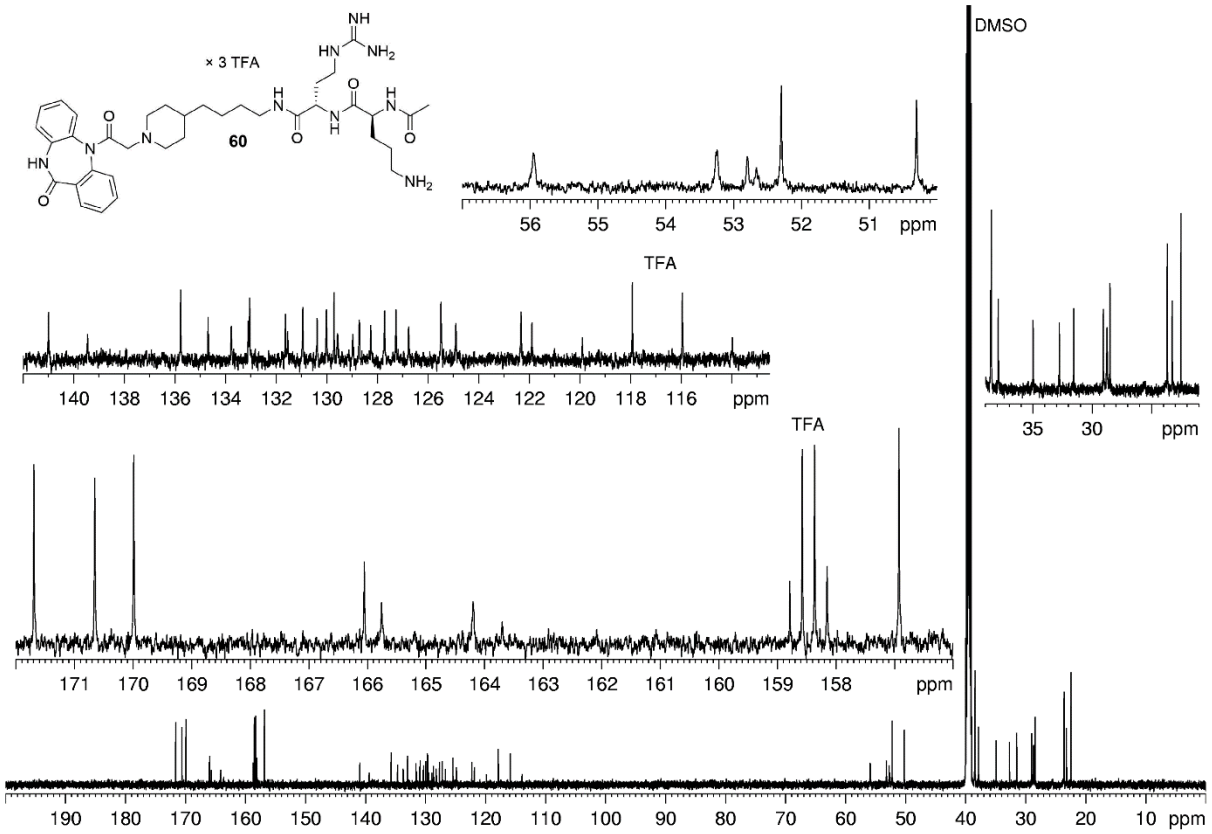
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **59**



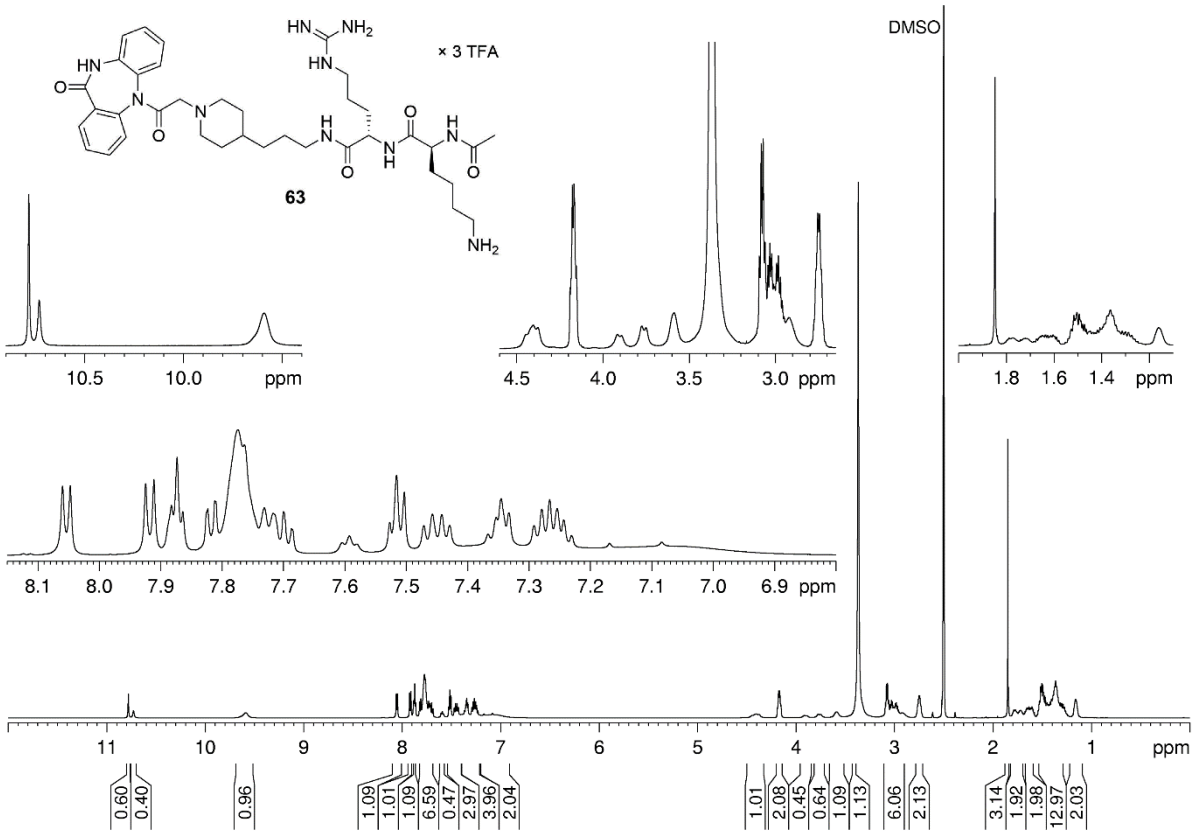
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **60**



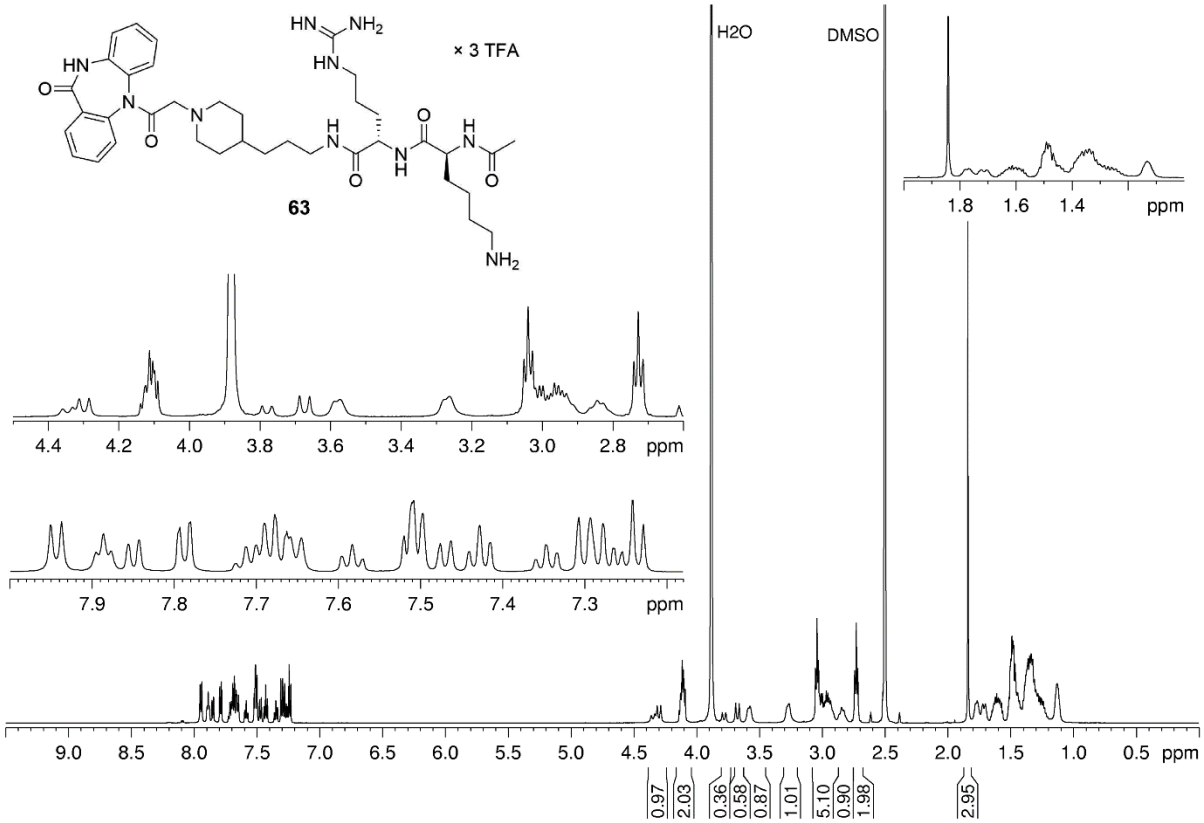
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **60**



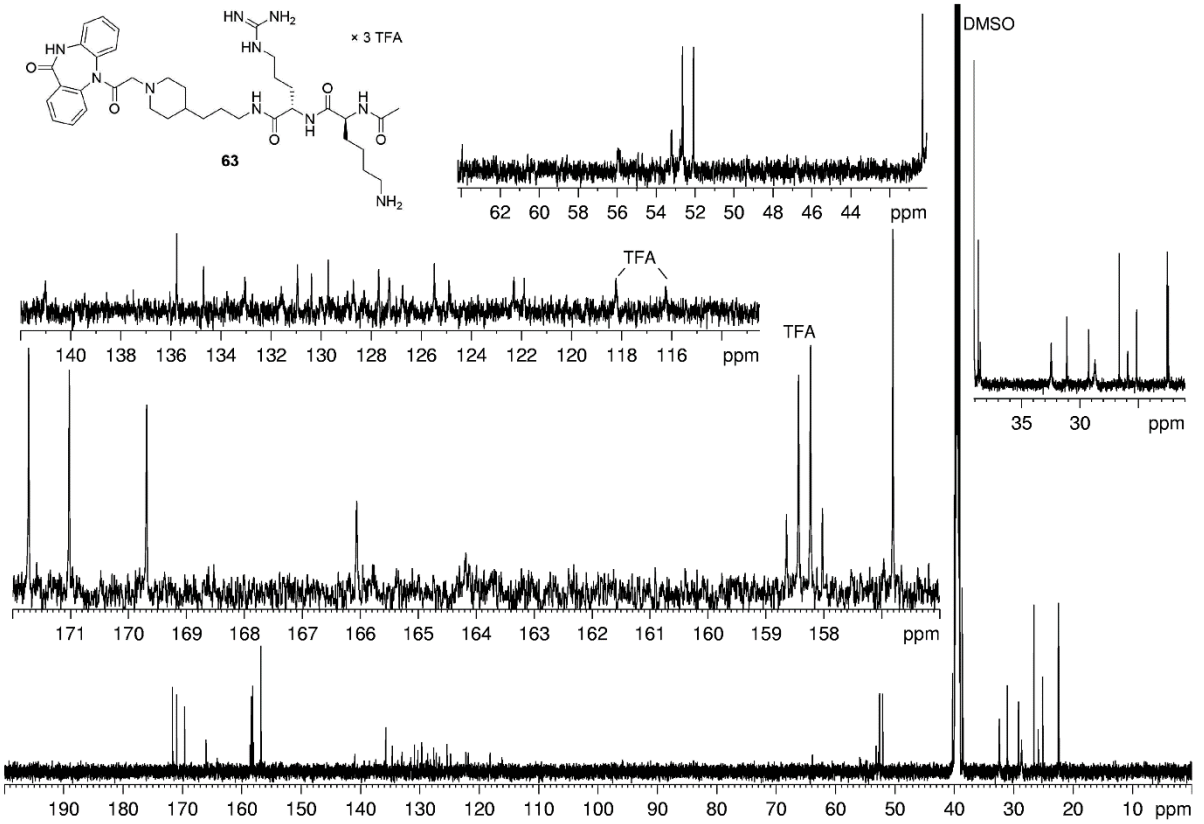
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **60**



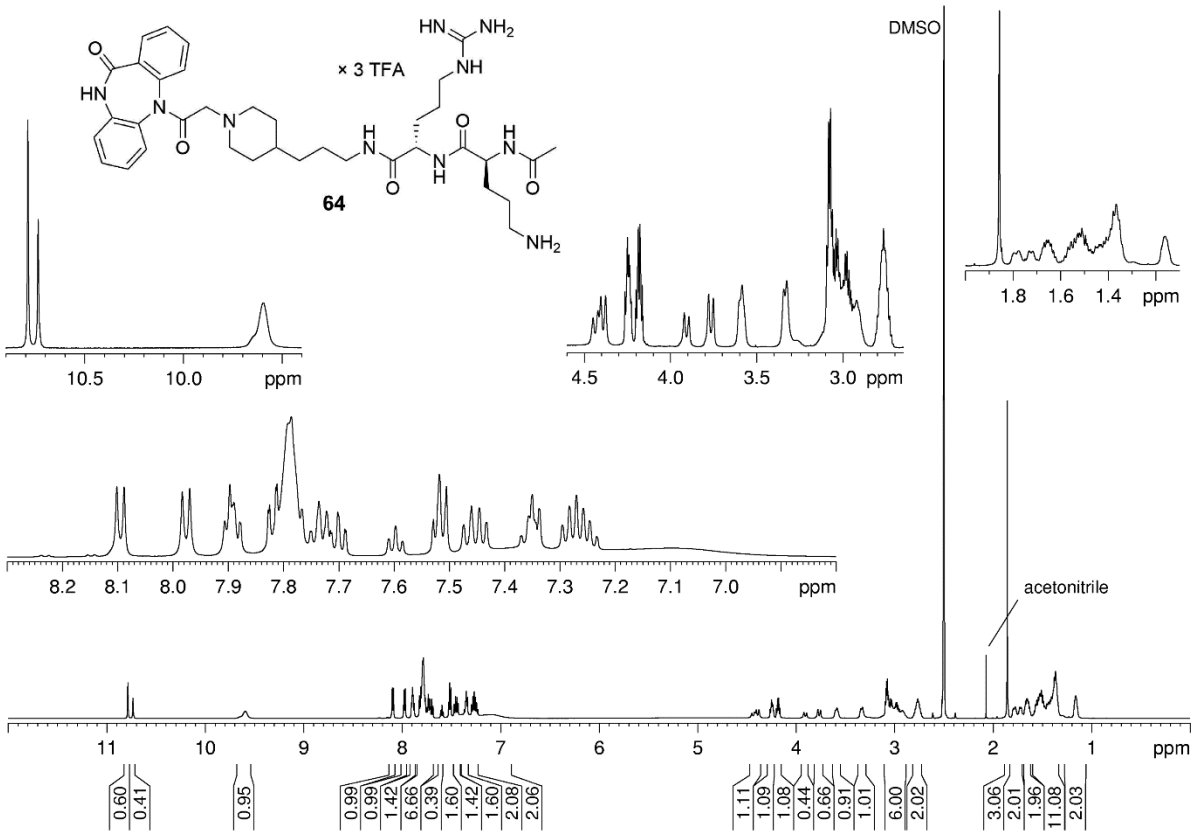
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **63**



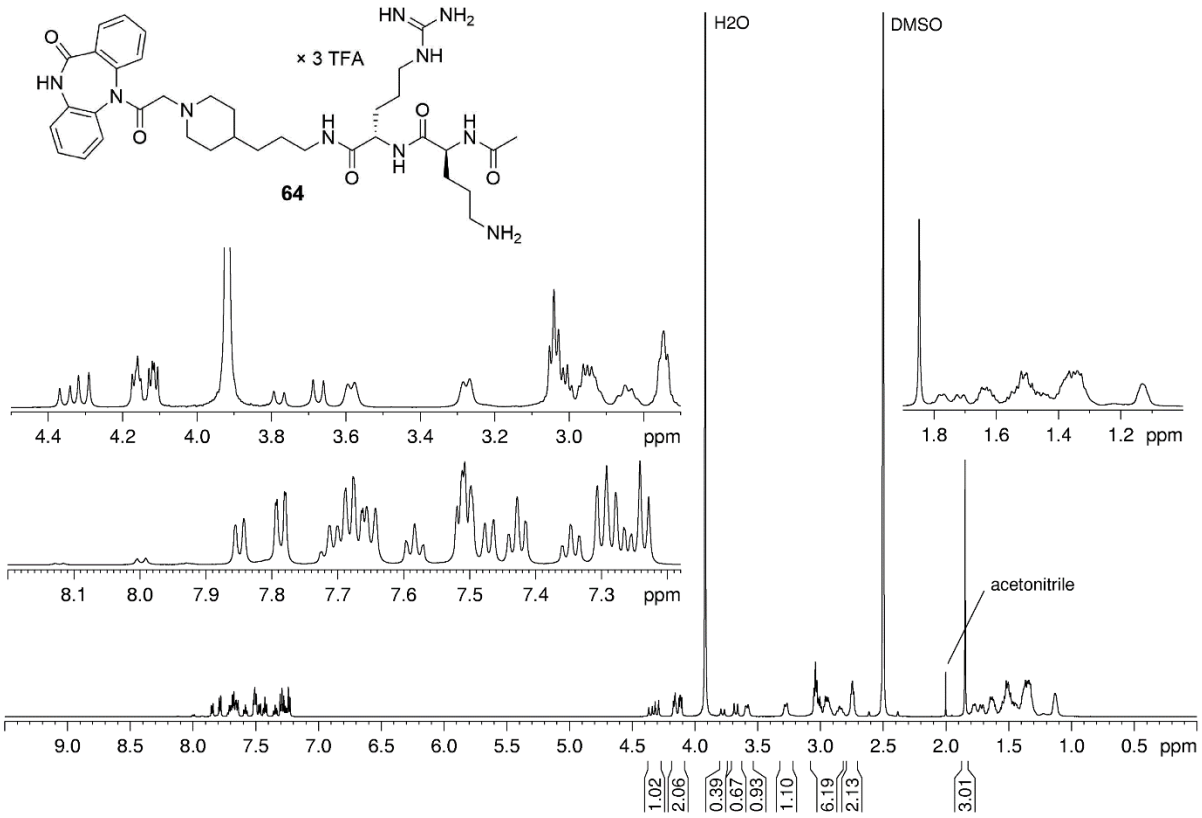
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **63**



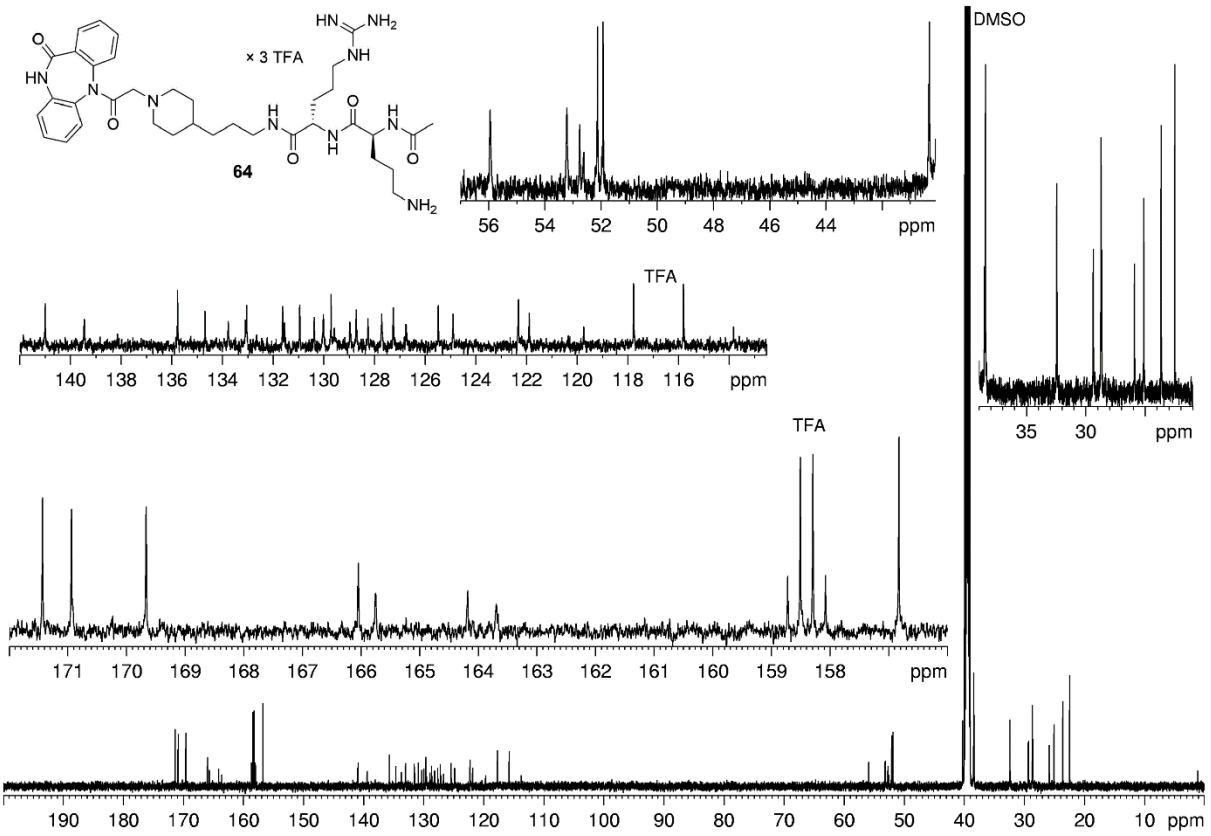
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **63**



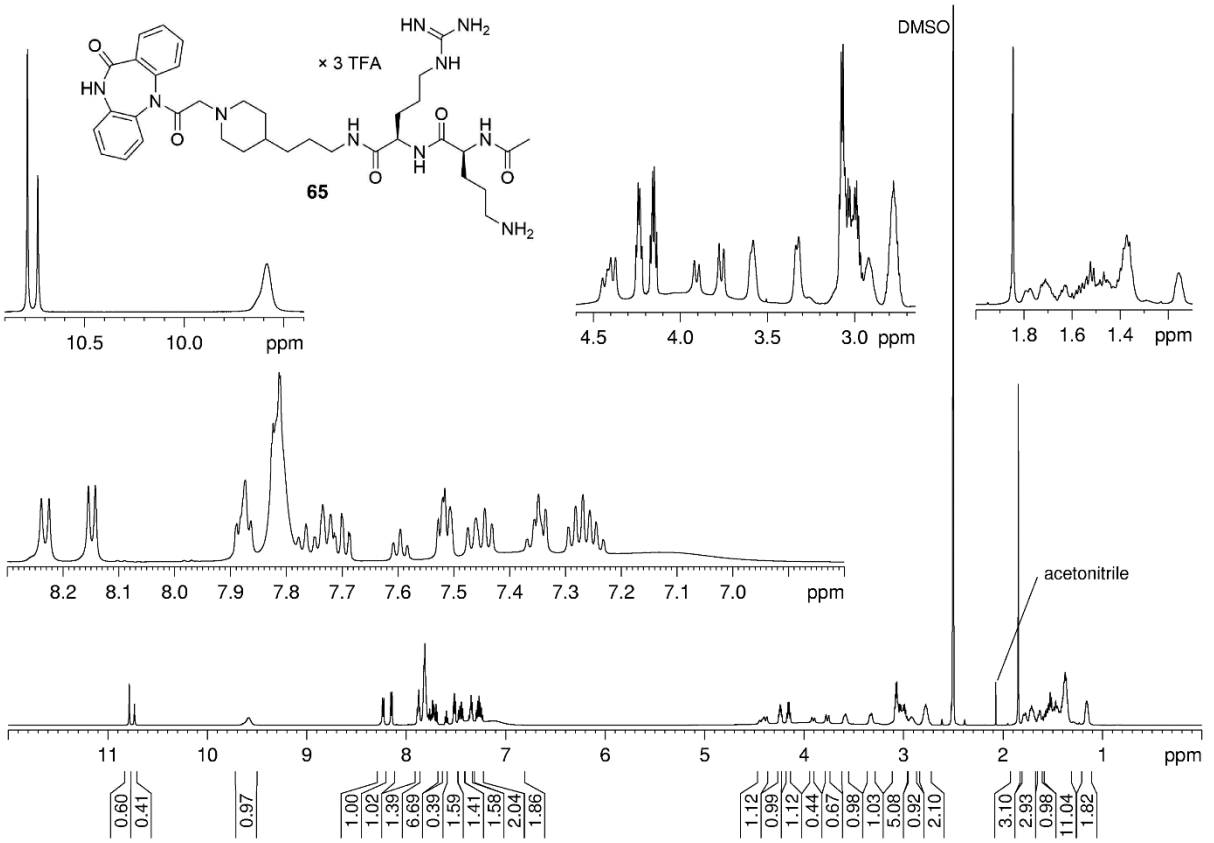
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **64**



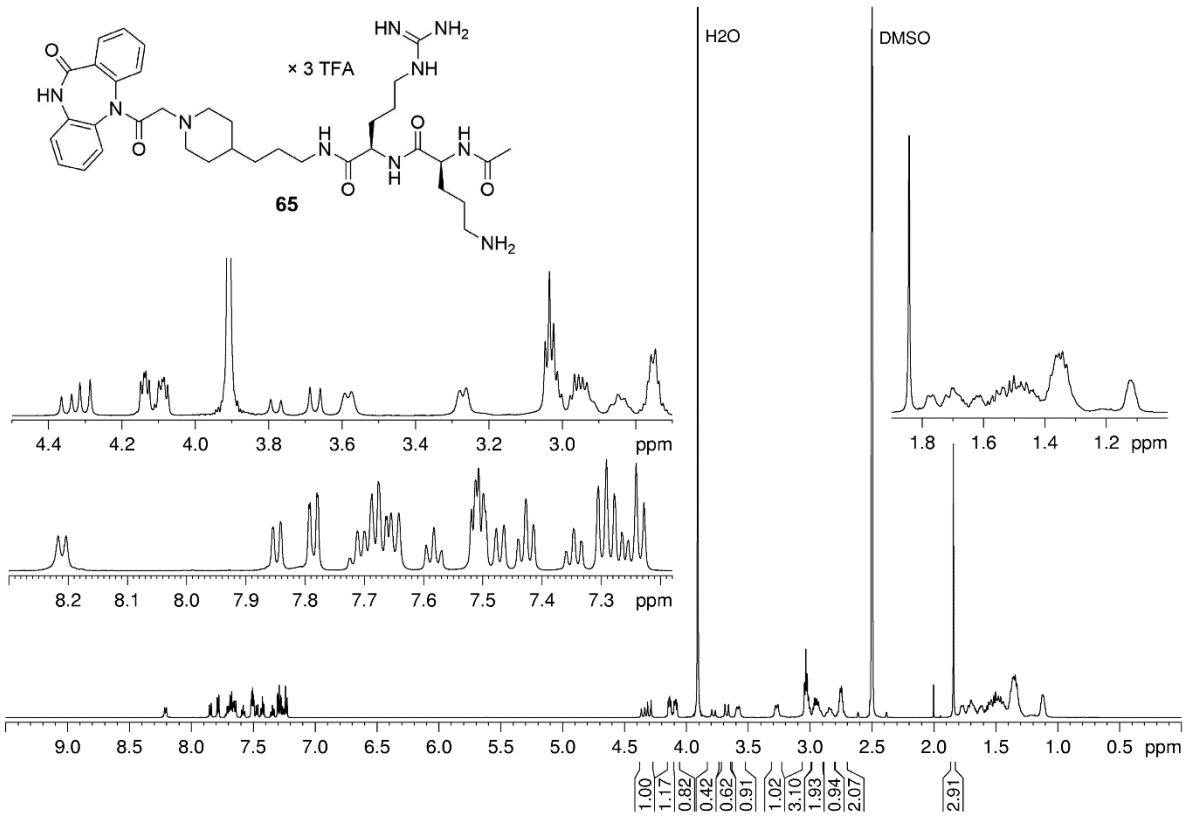
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **64**



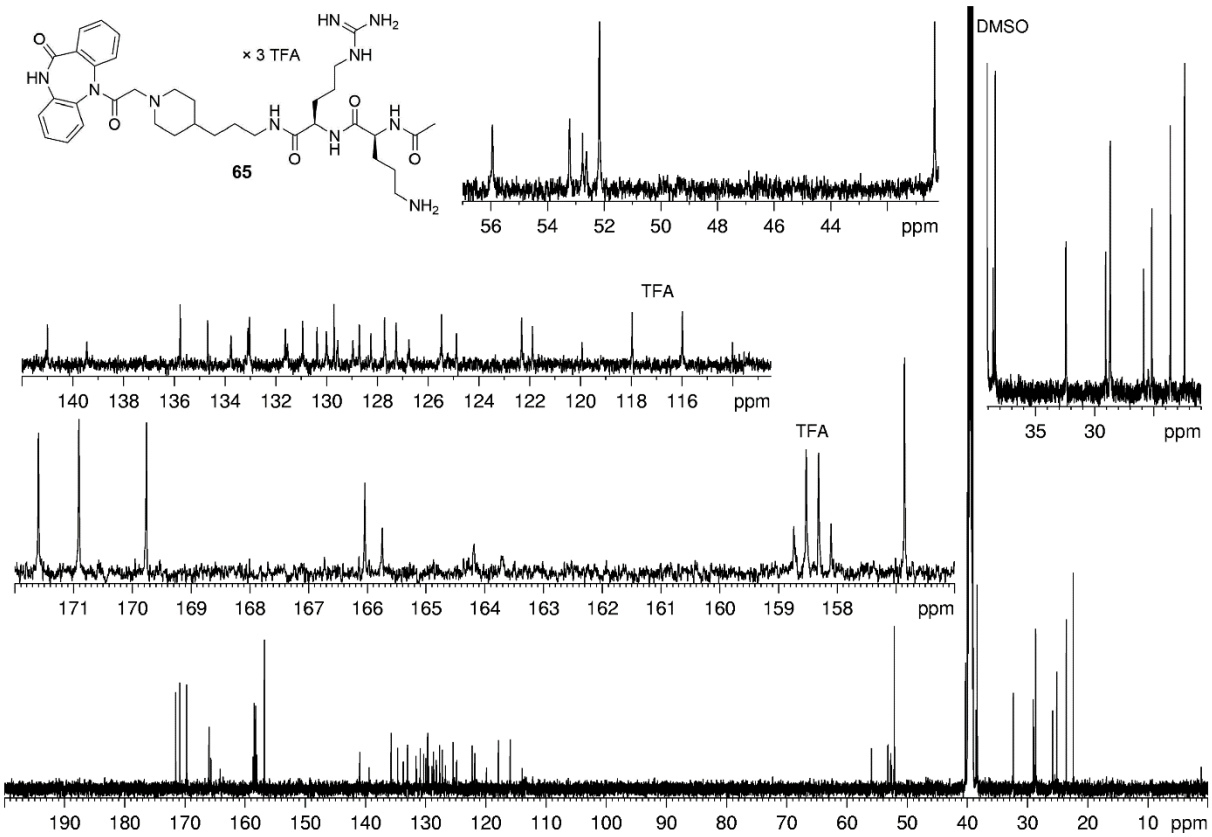
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **64**



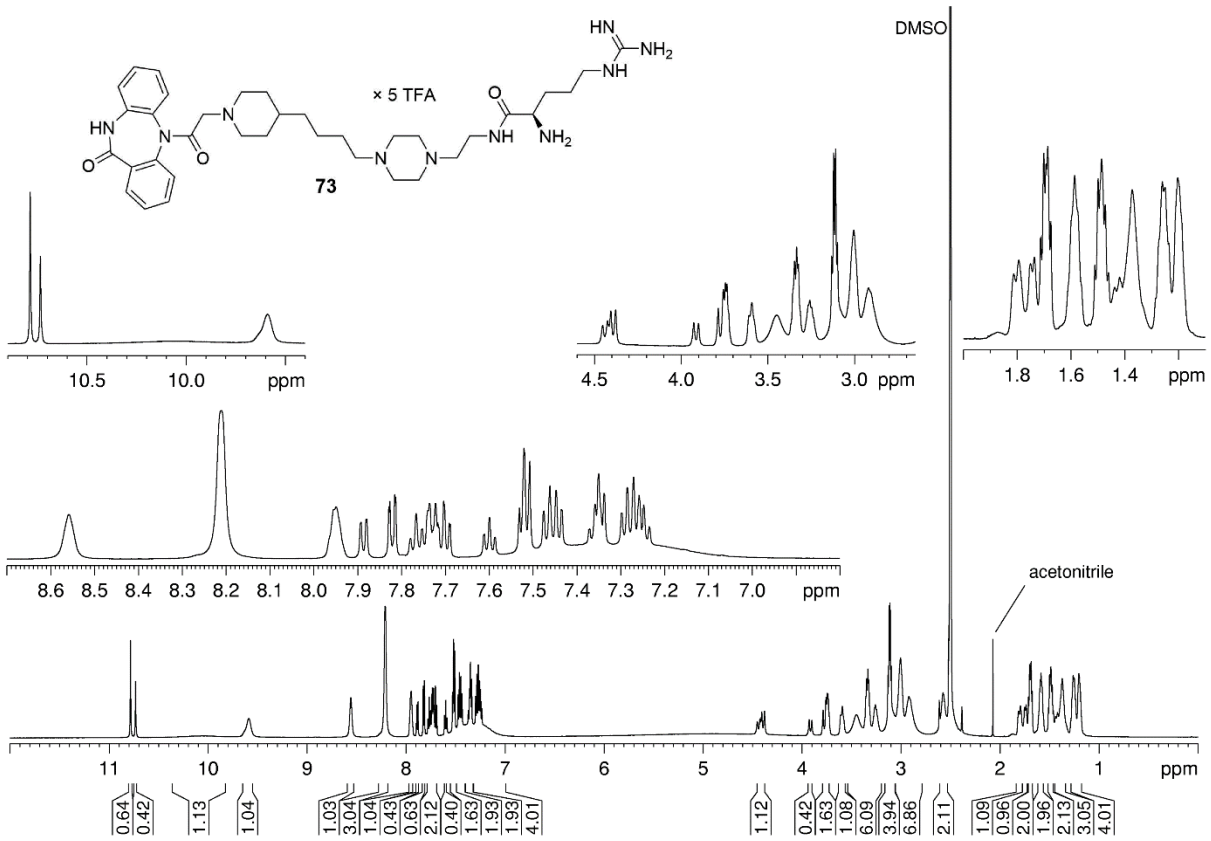
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **65**



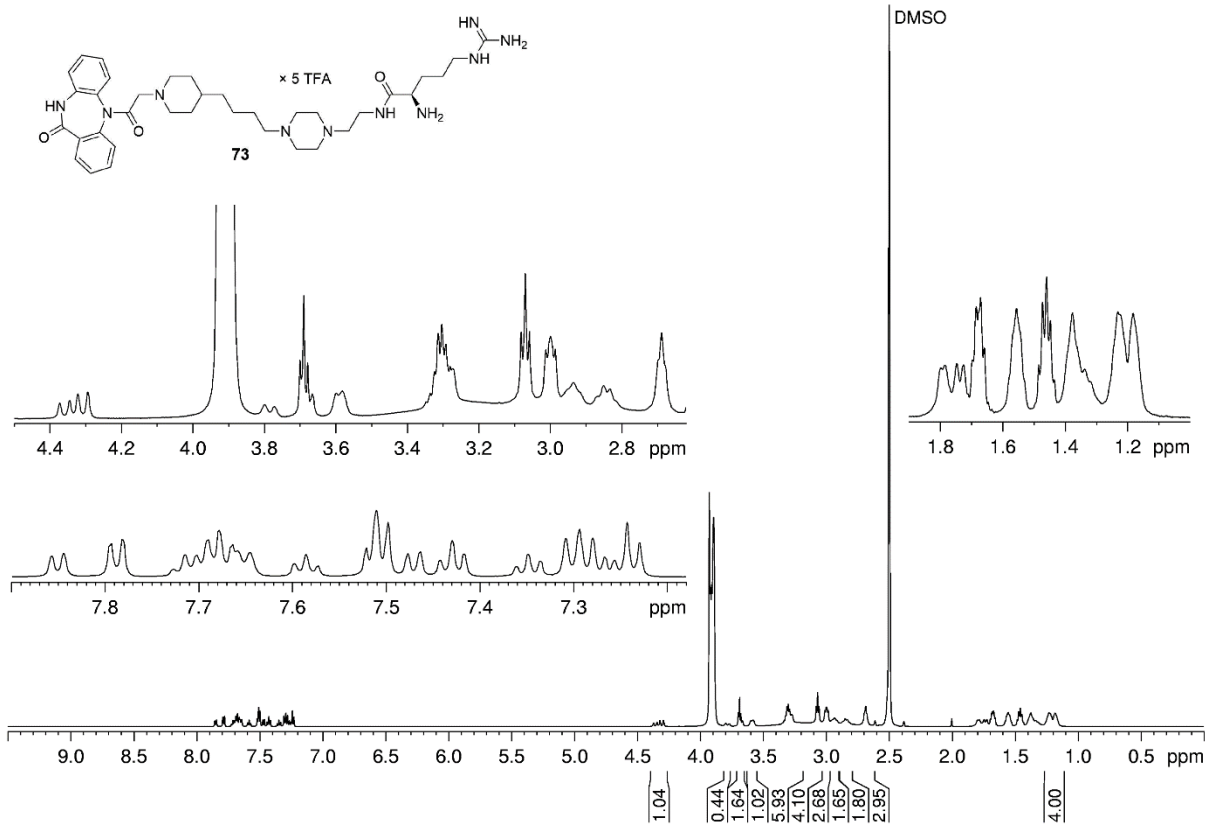
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **65**



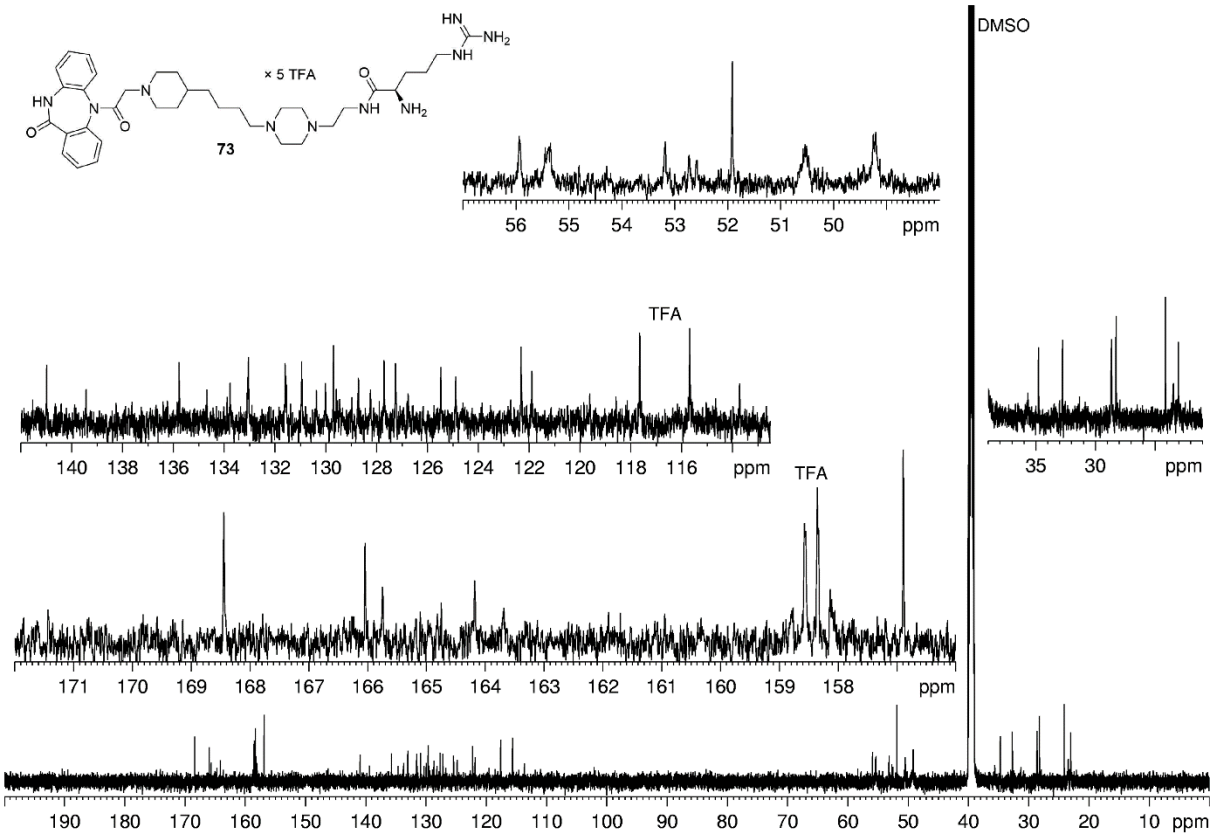
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **65**



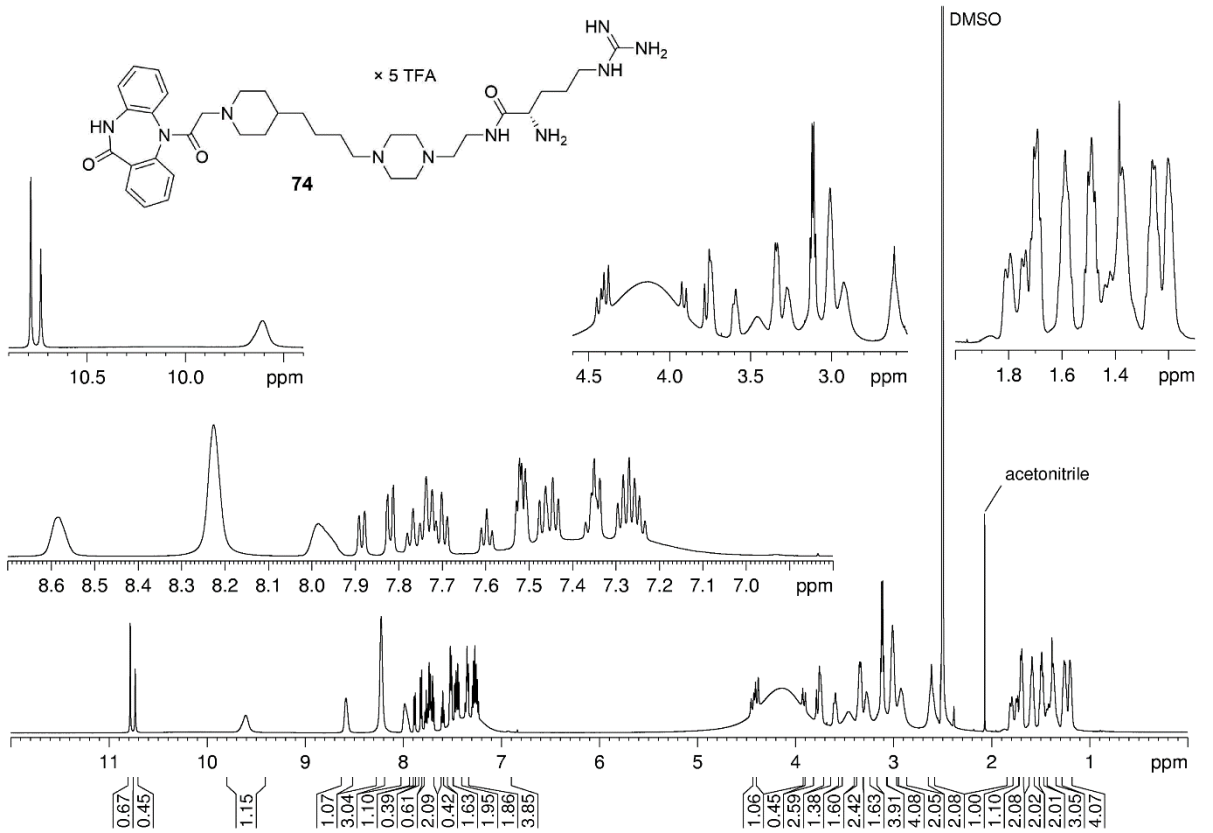
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **73**



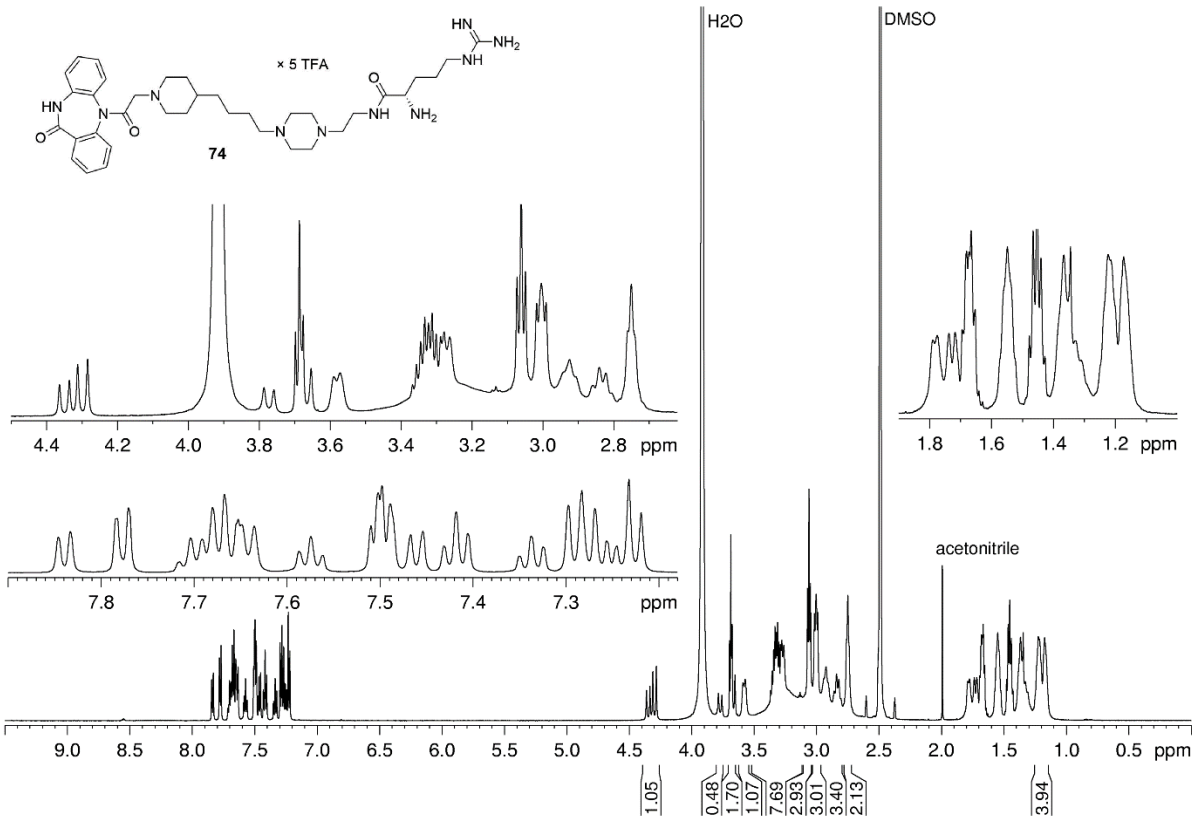
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **73**



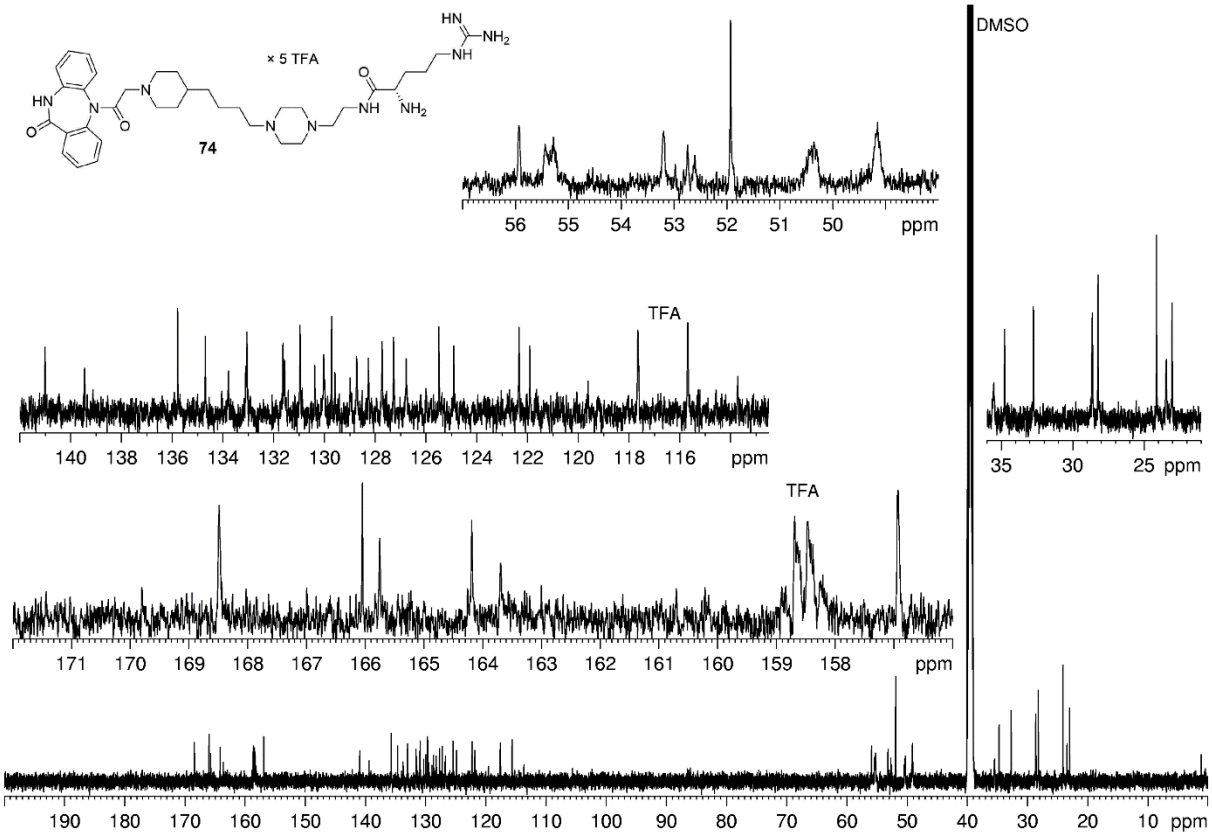
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **73**



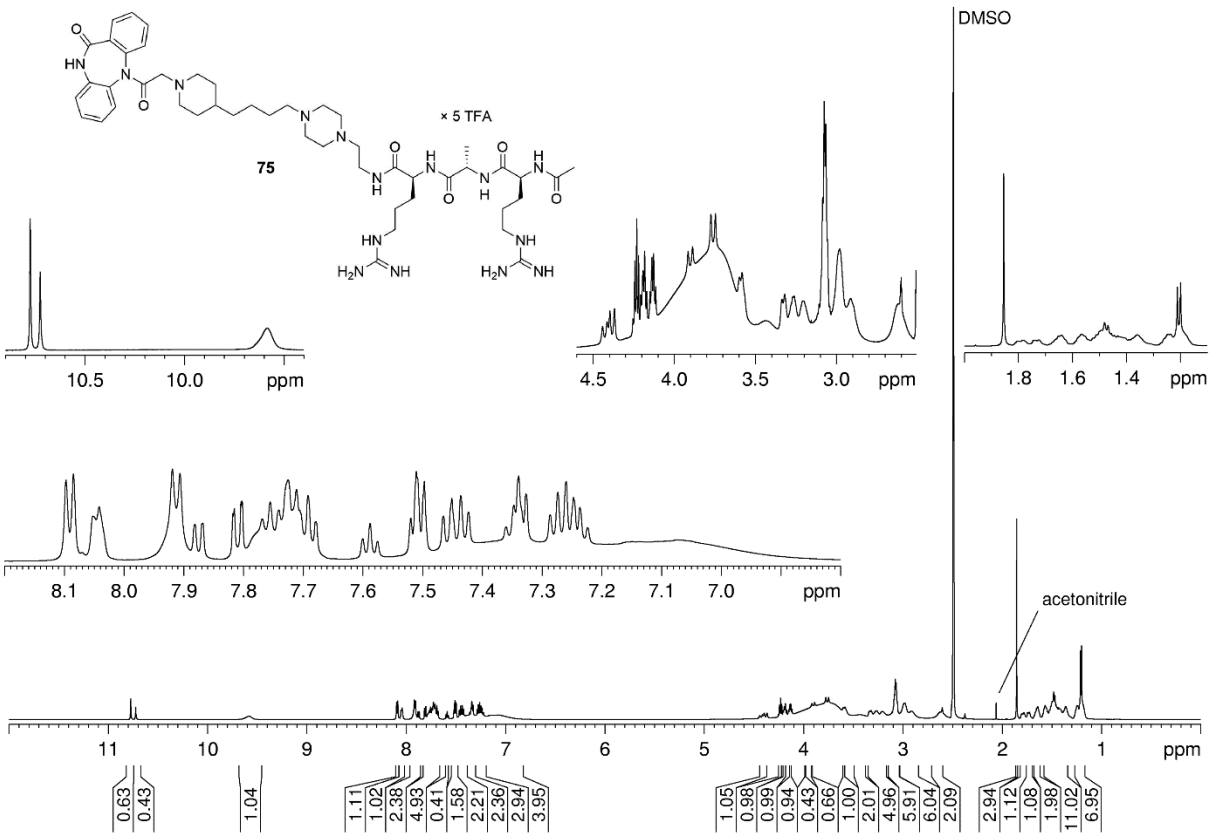
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **74**



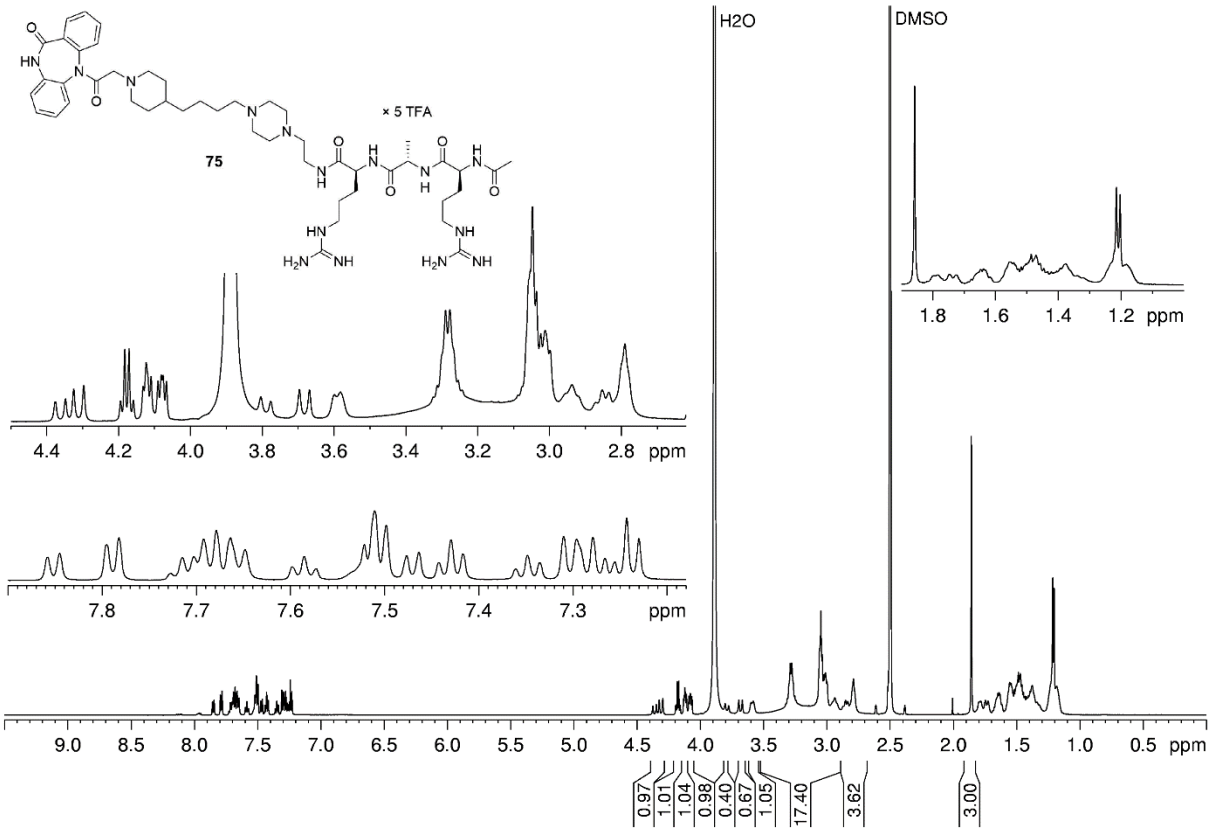
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **74**



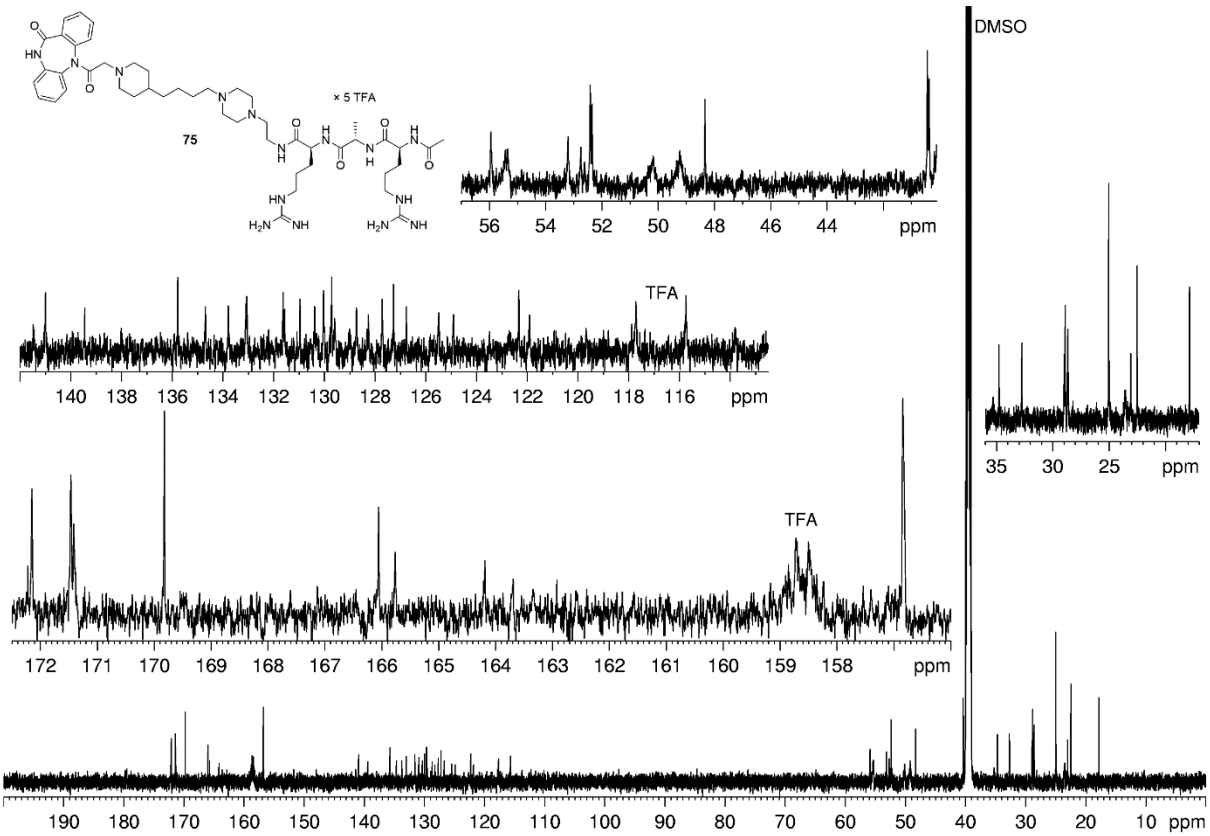
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **74**



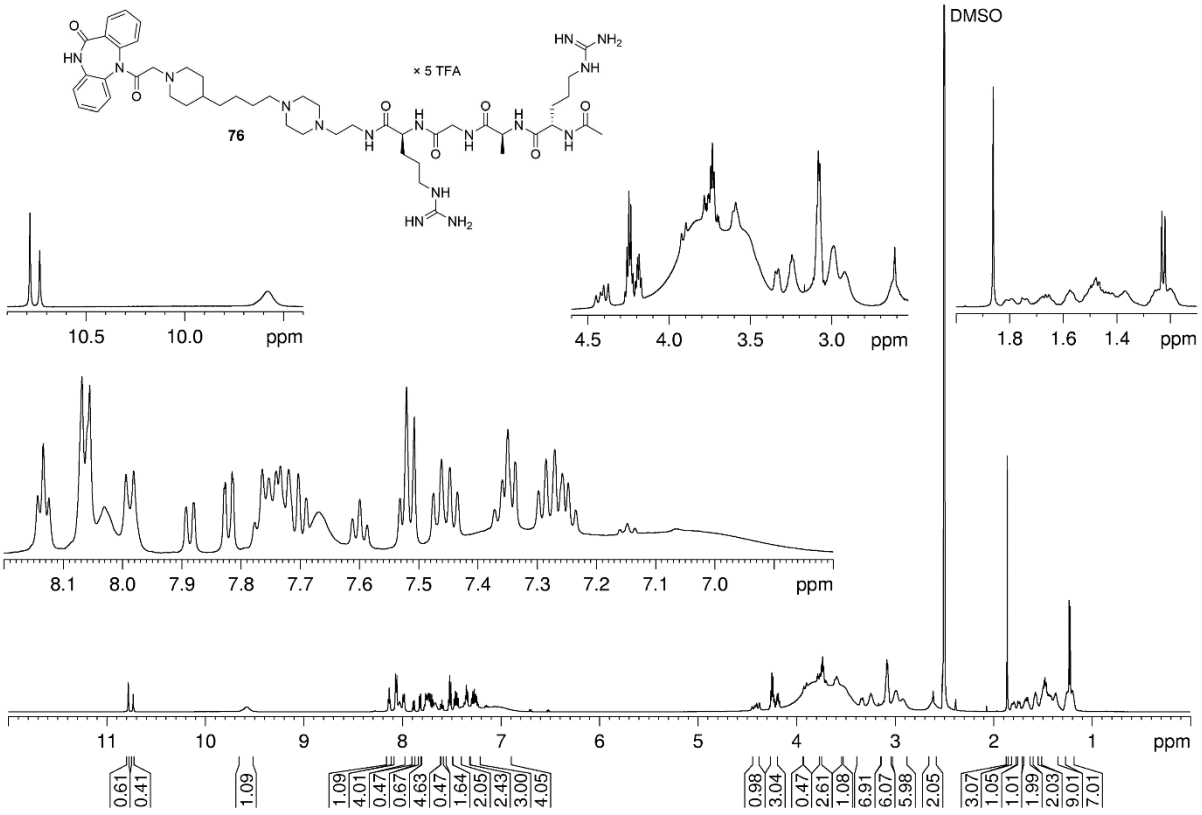
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **75**



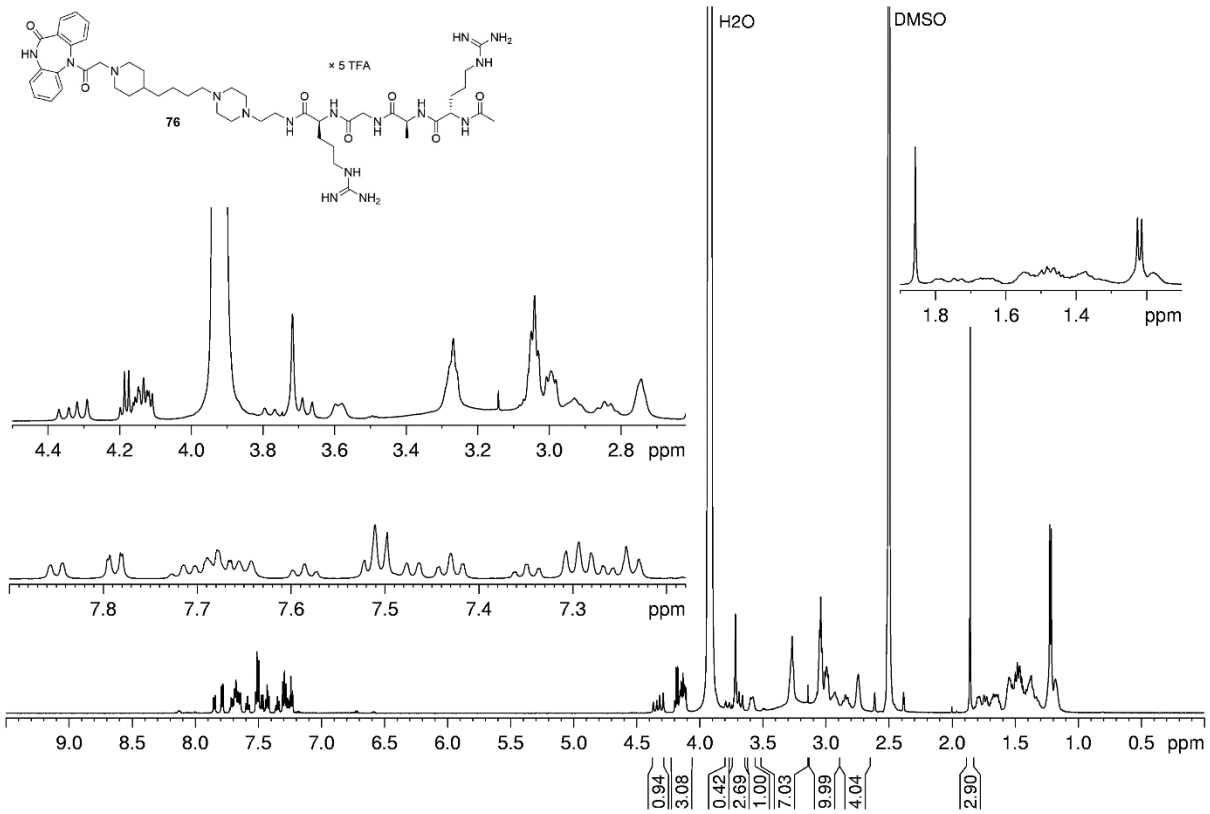
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **75**



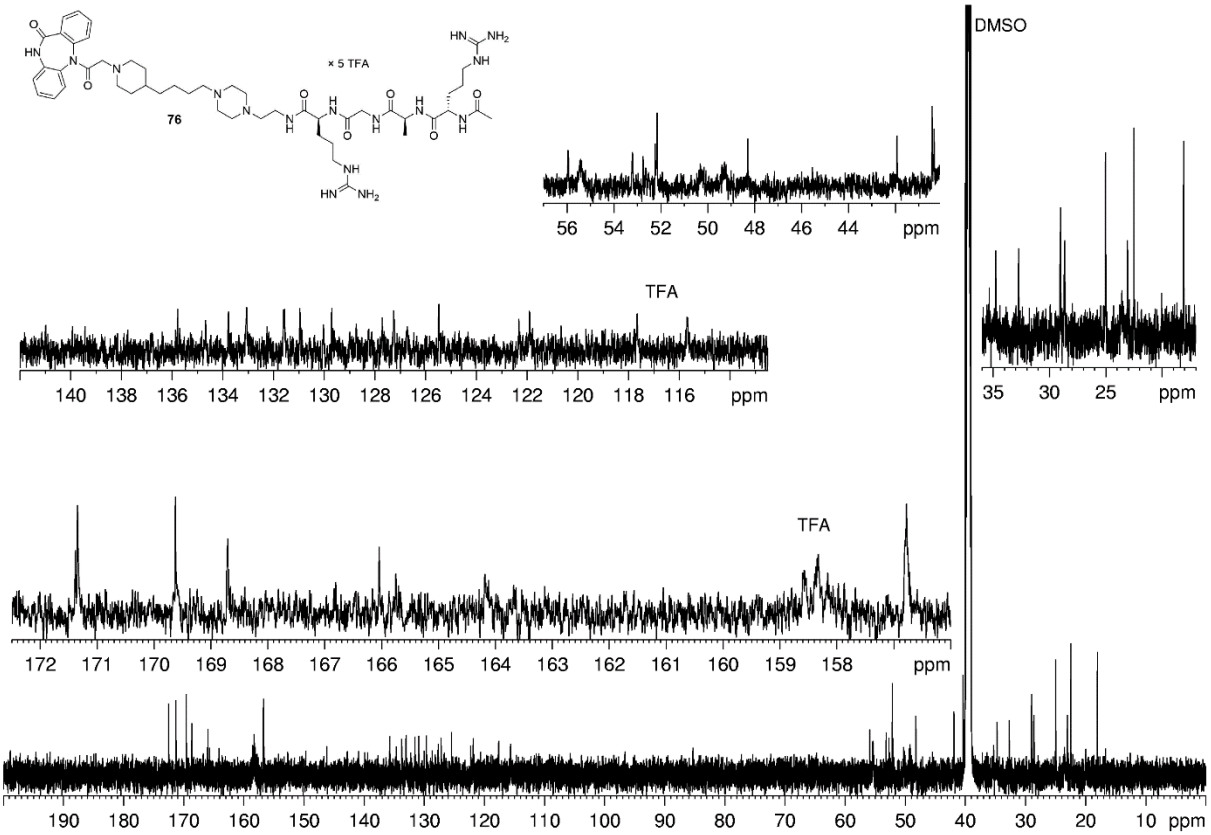
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **75**



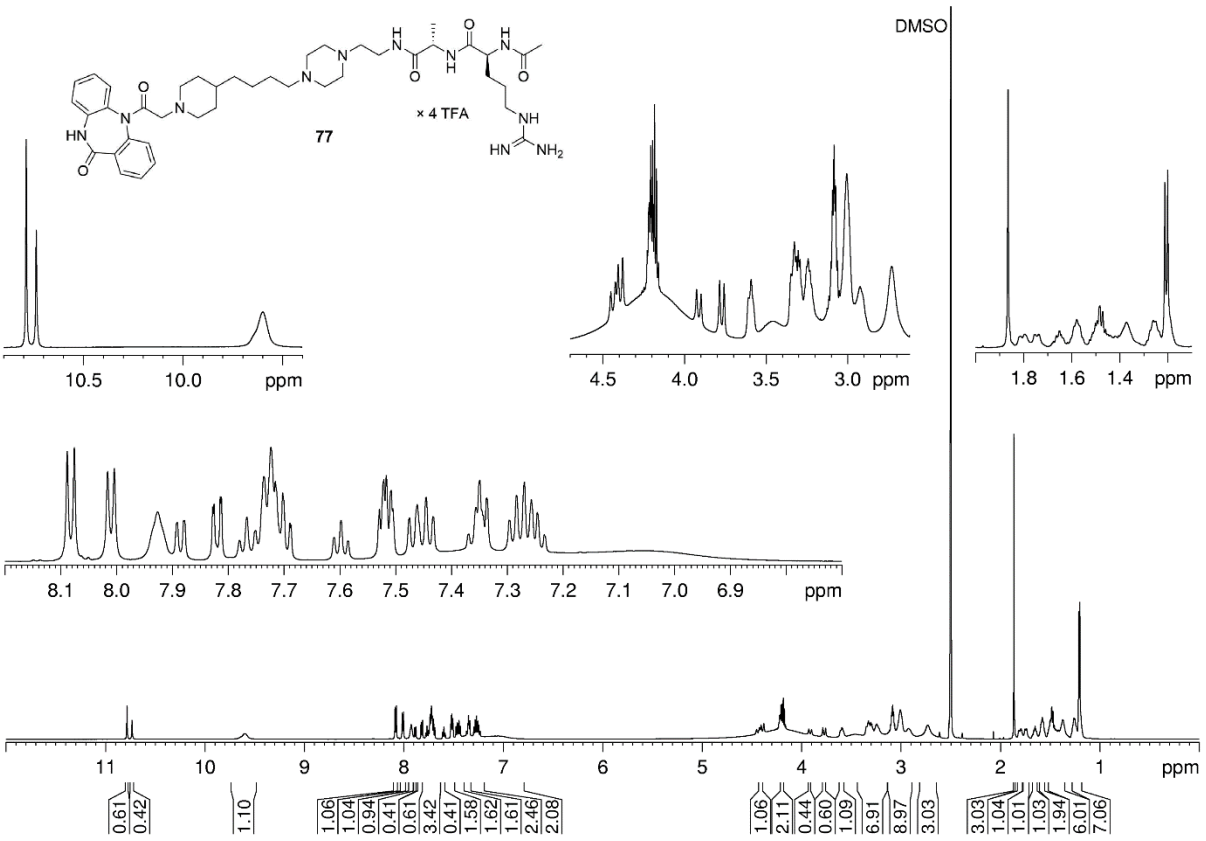
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **76**



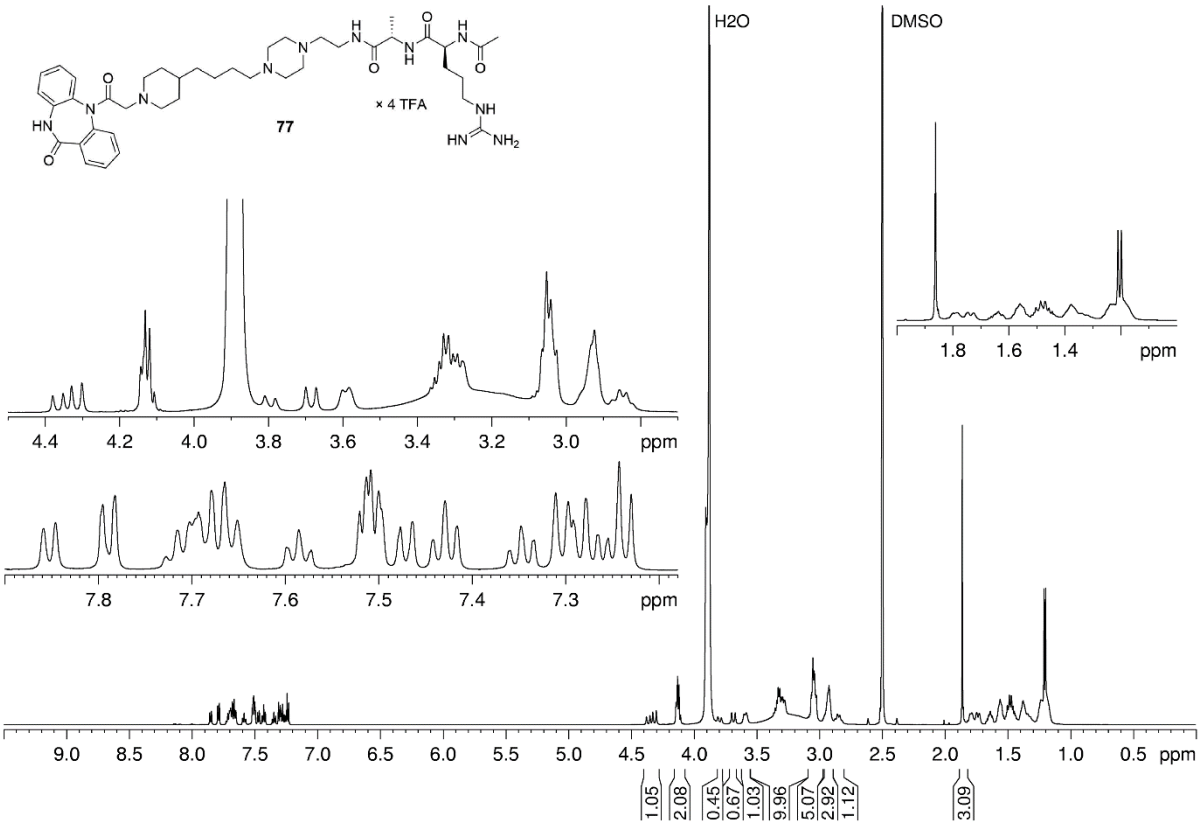
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **76**



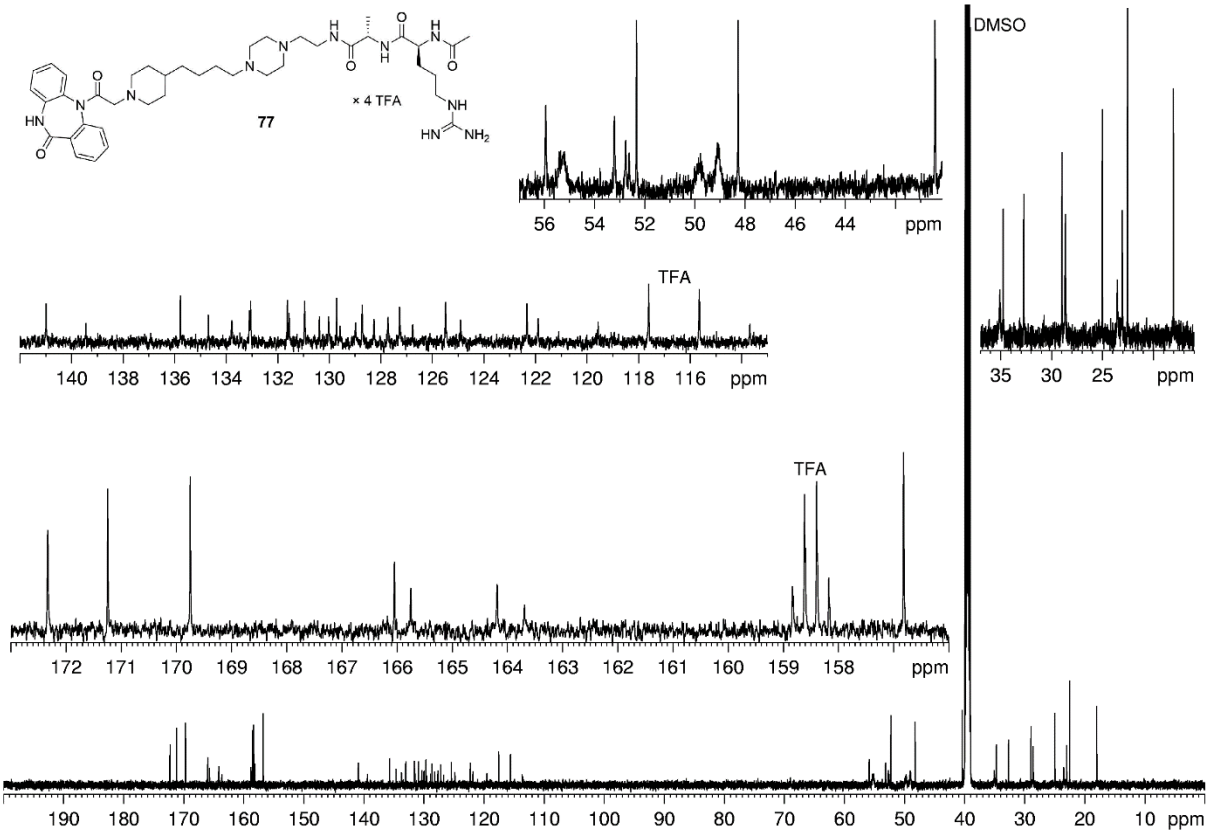
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **76**



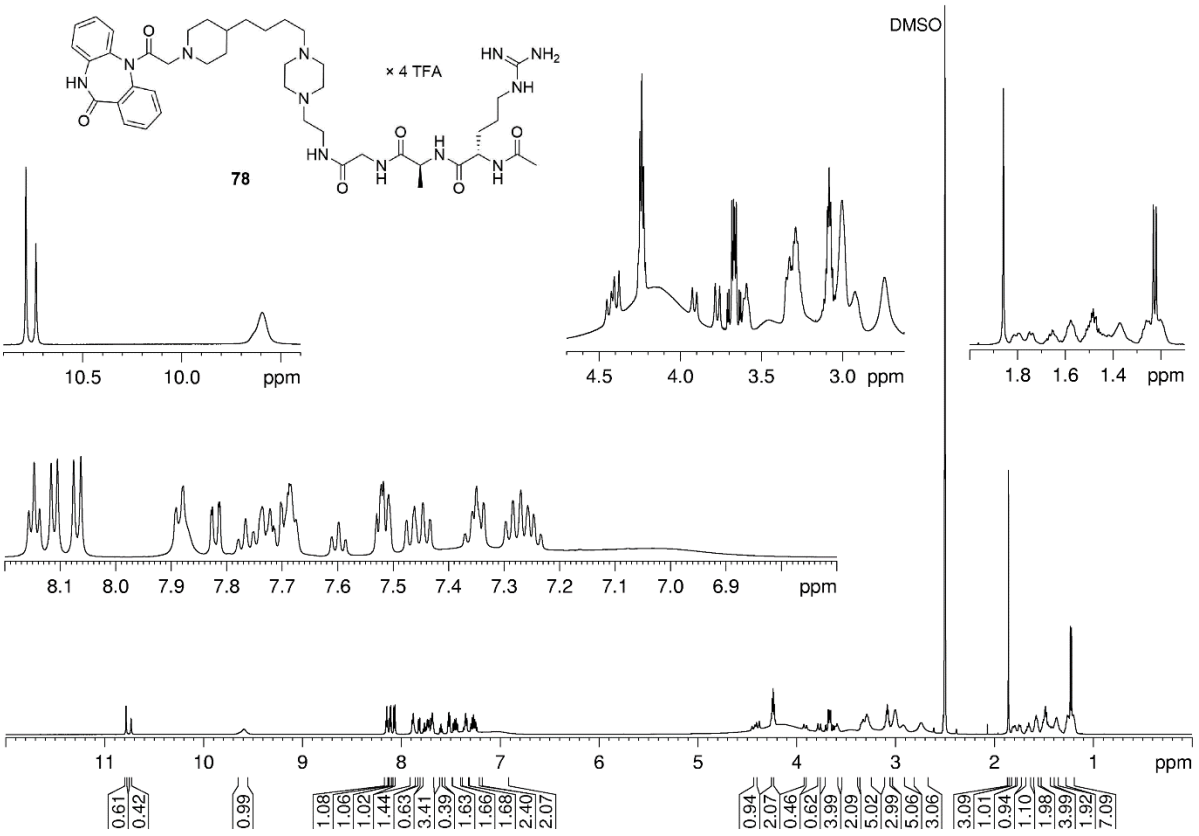
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **77**



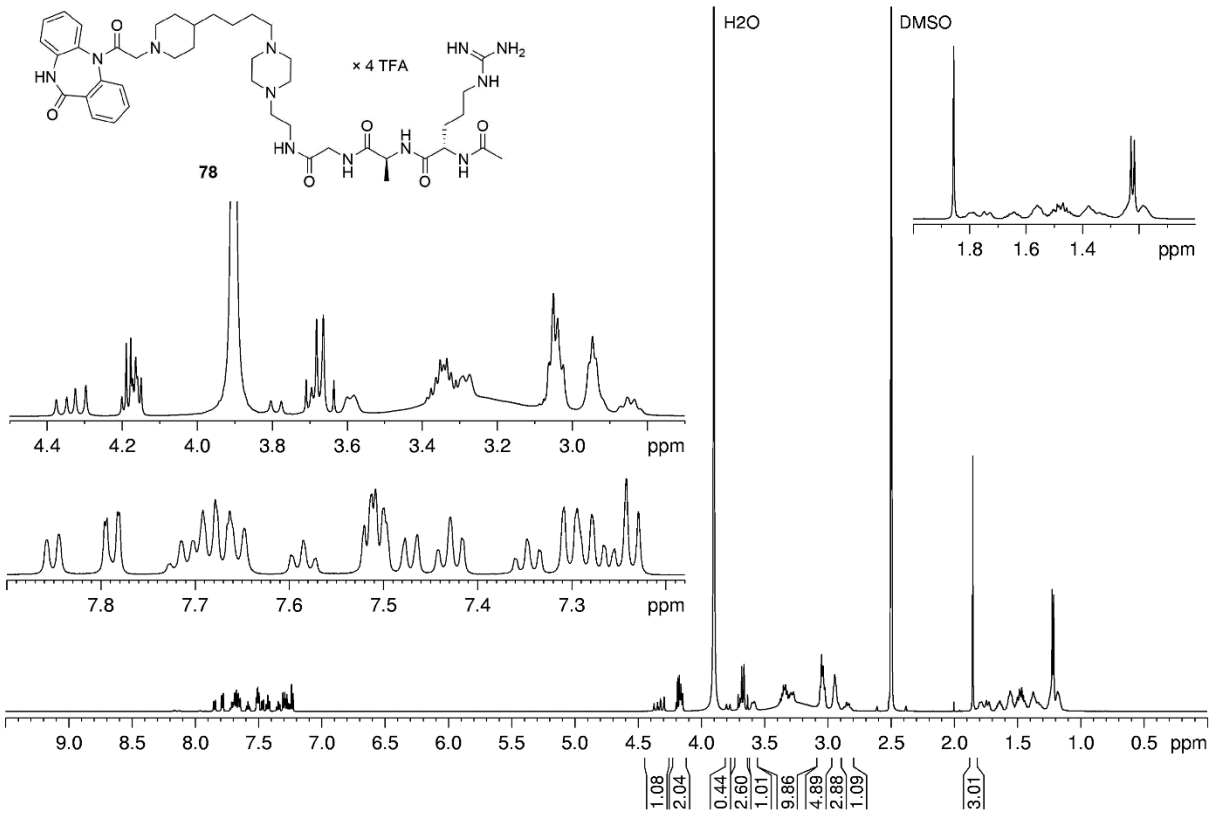
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **77**



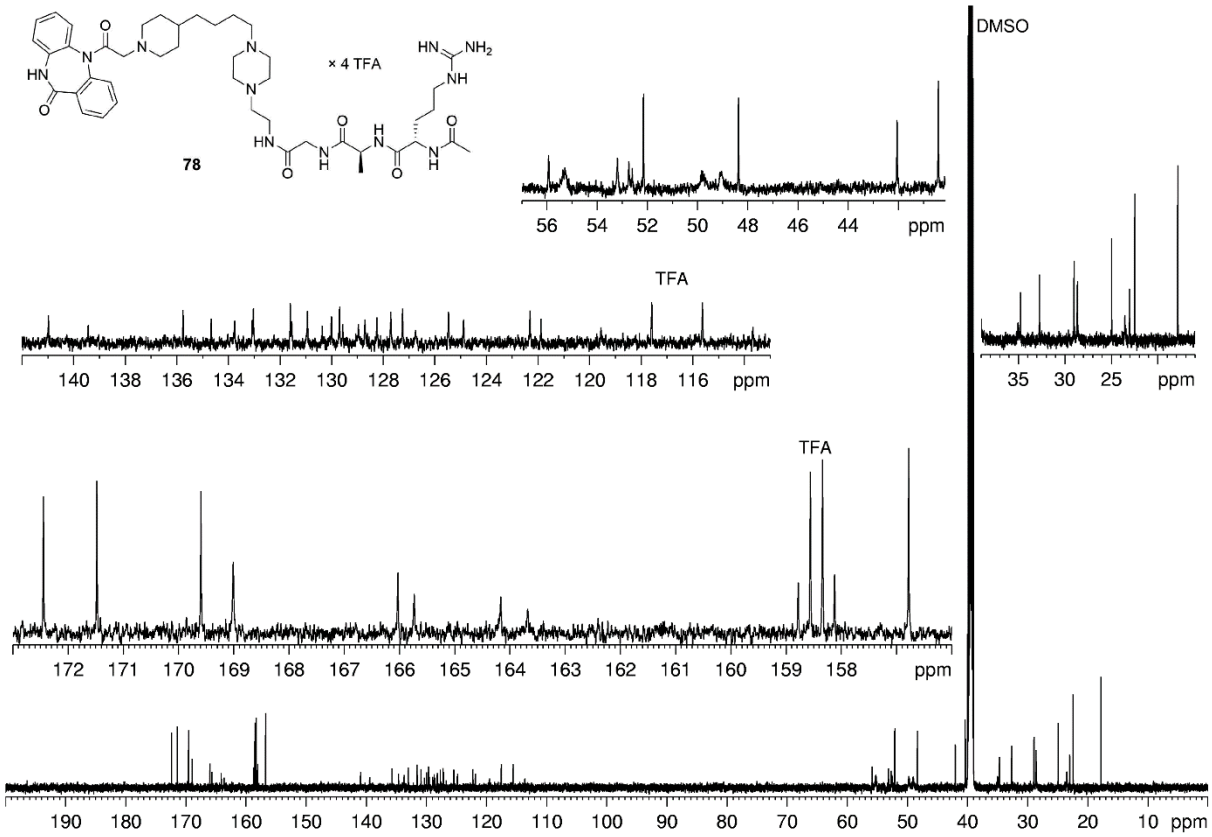
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **77**



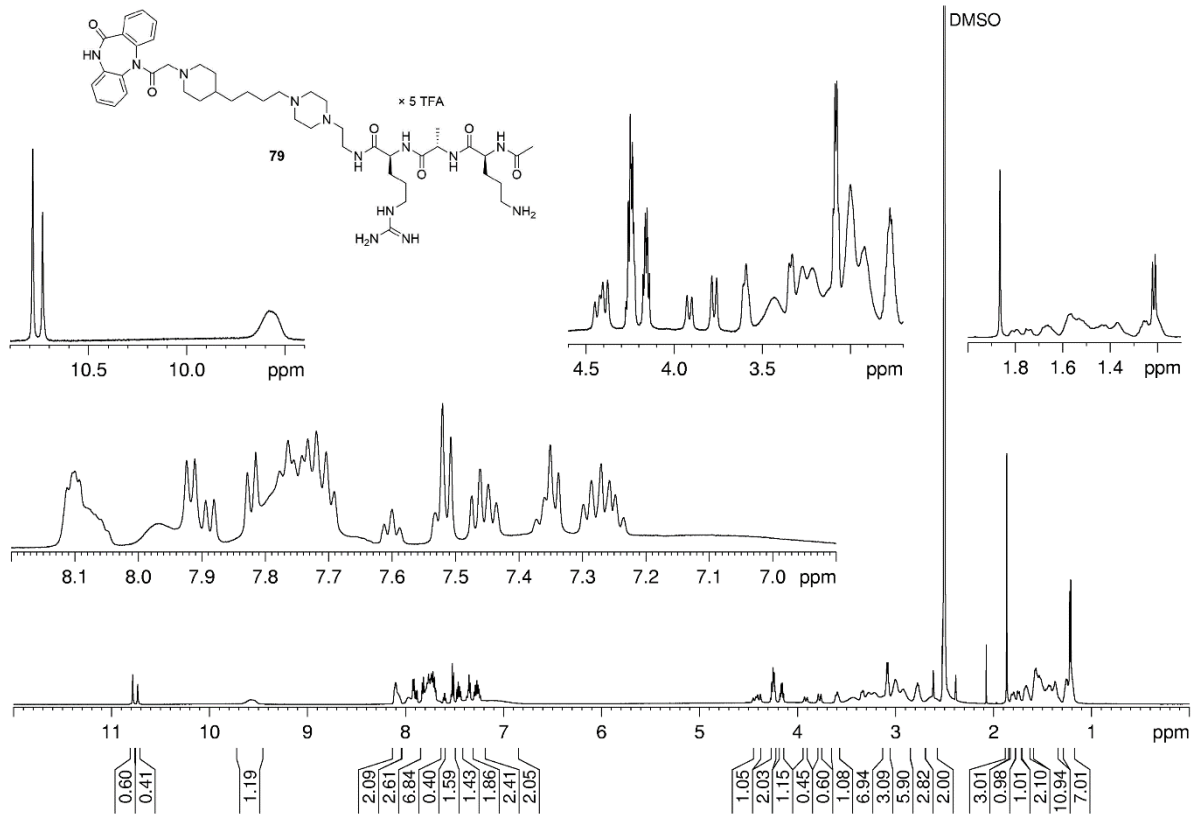
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **78**



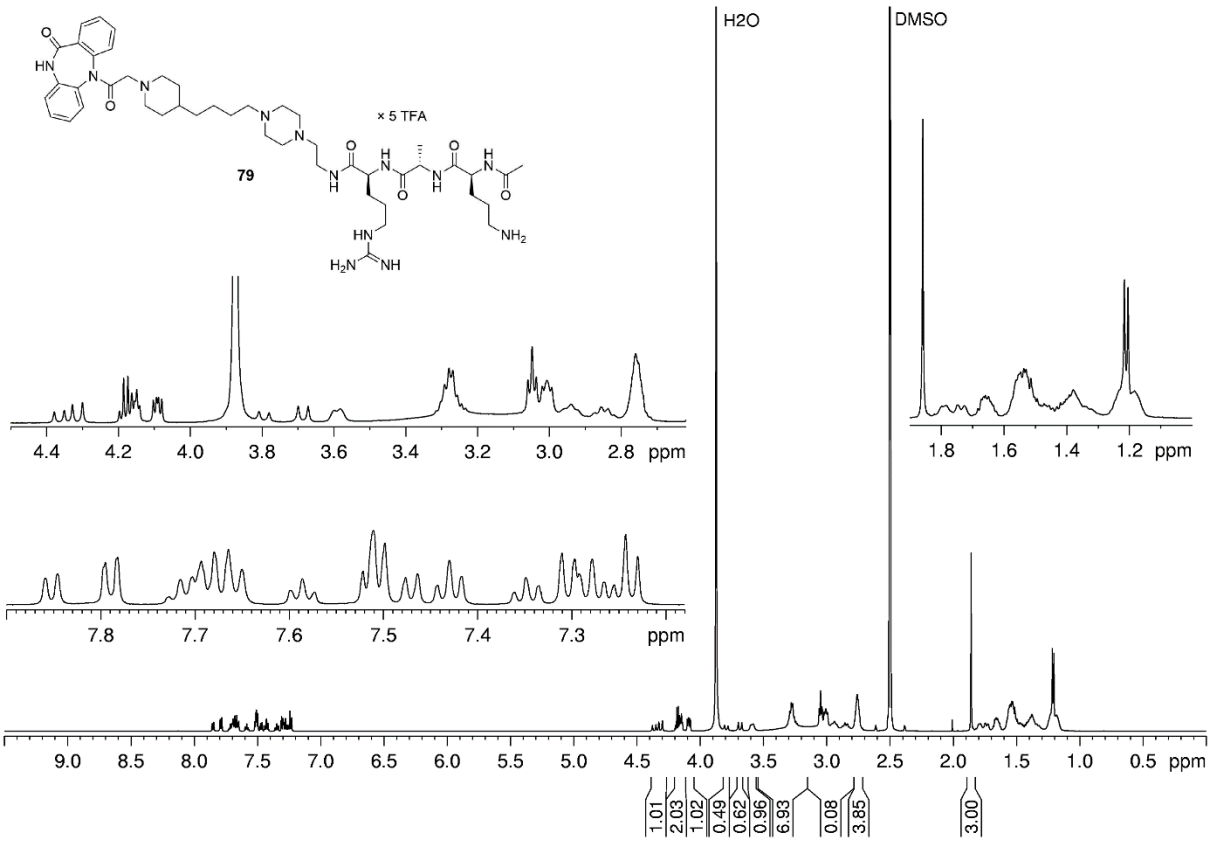
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **78**



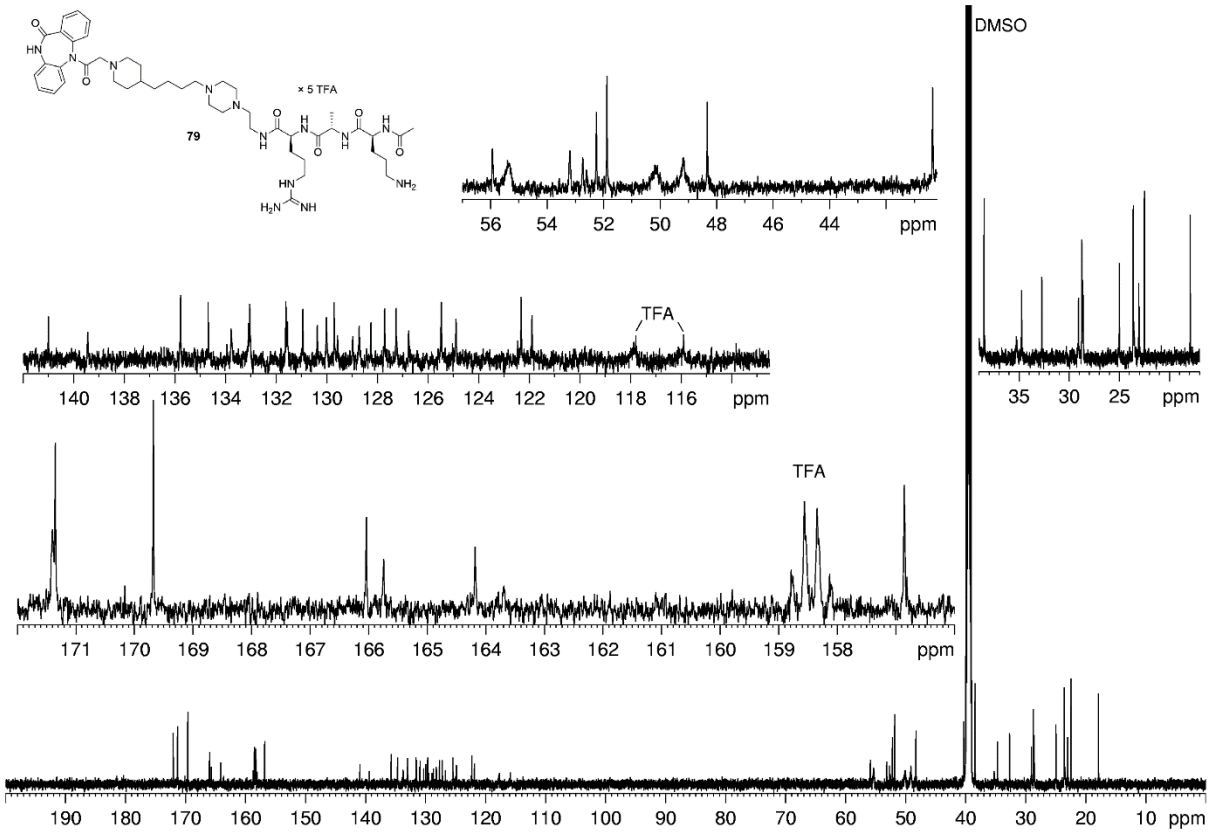
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **78**



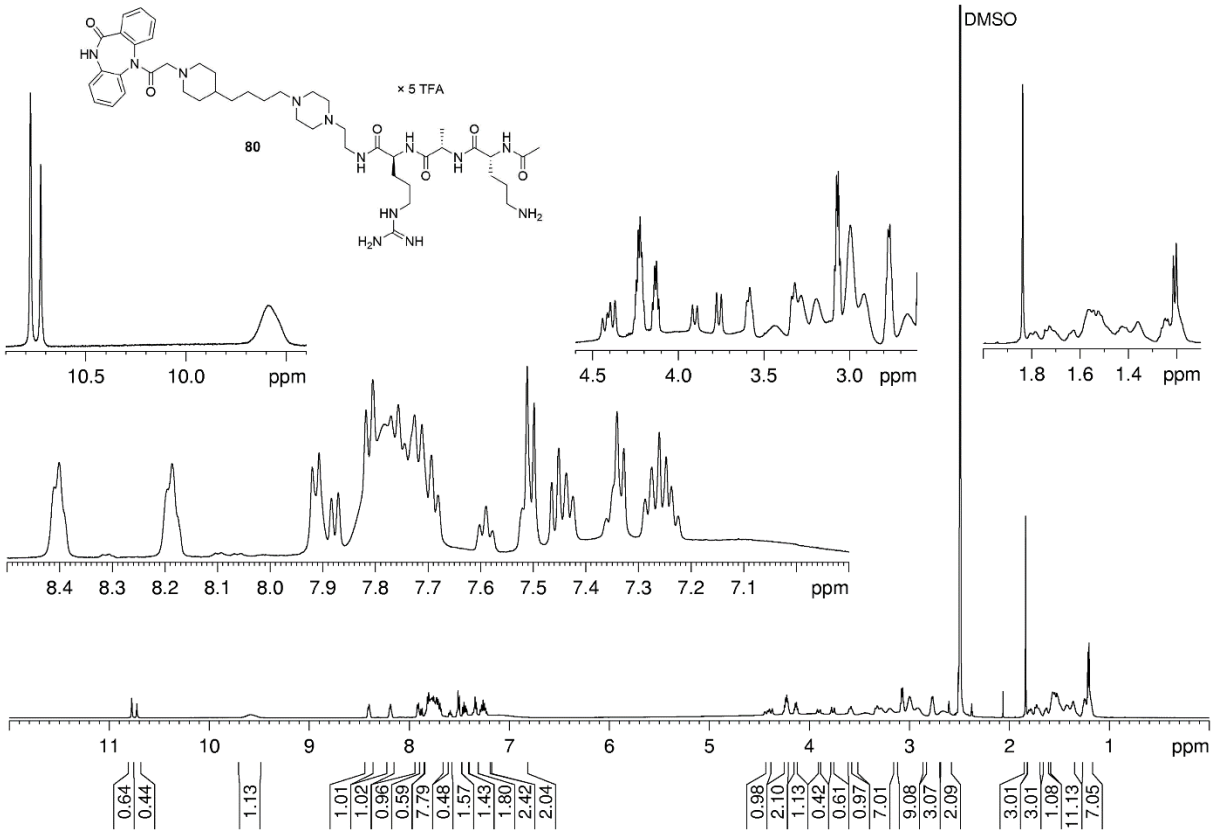
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **79**



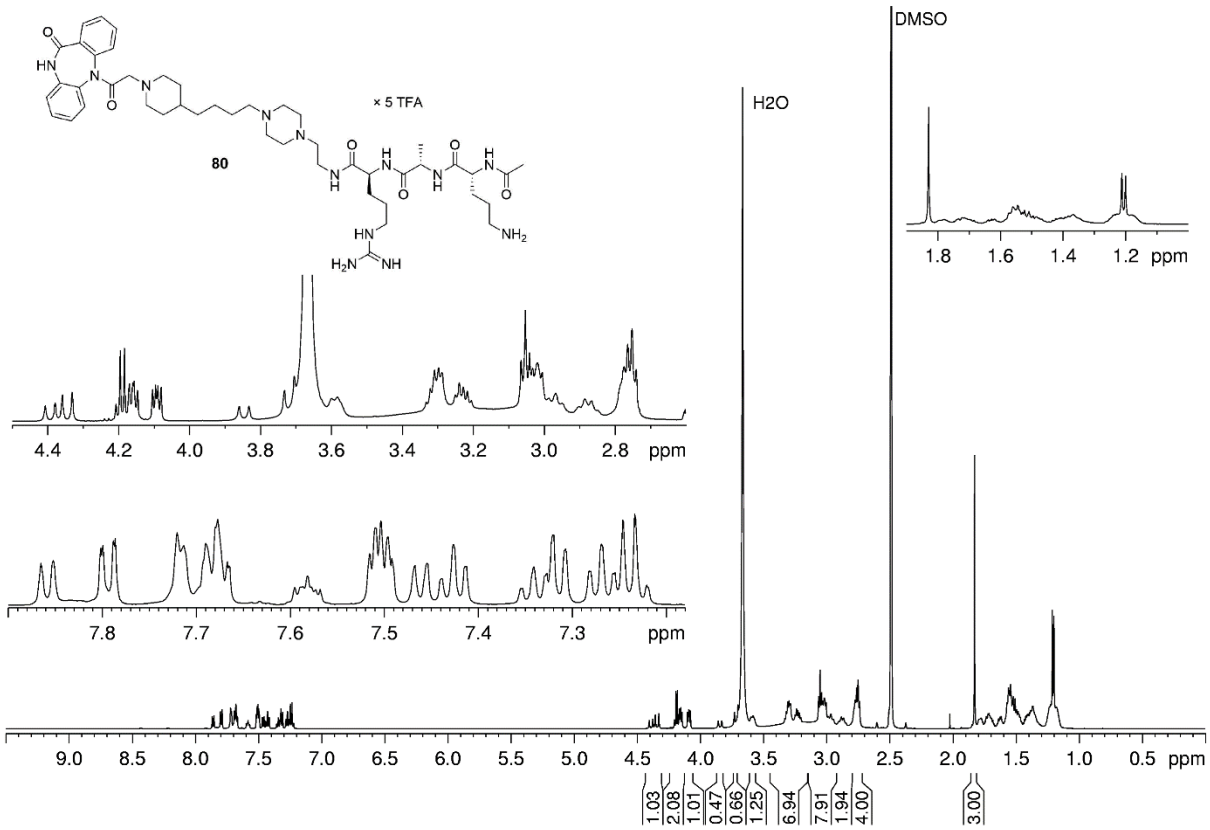
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **79**



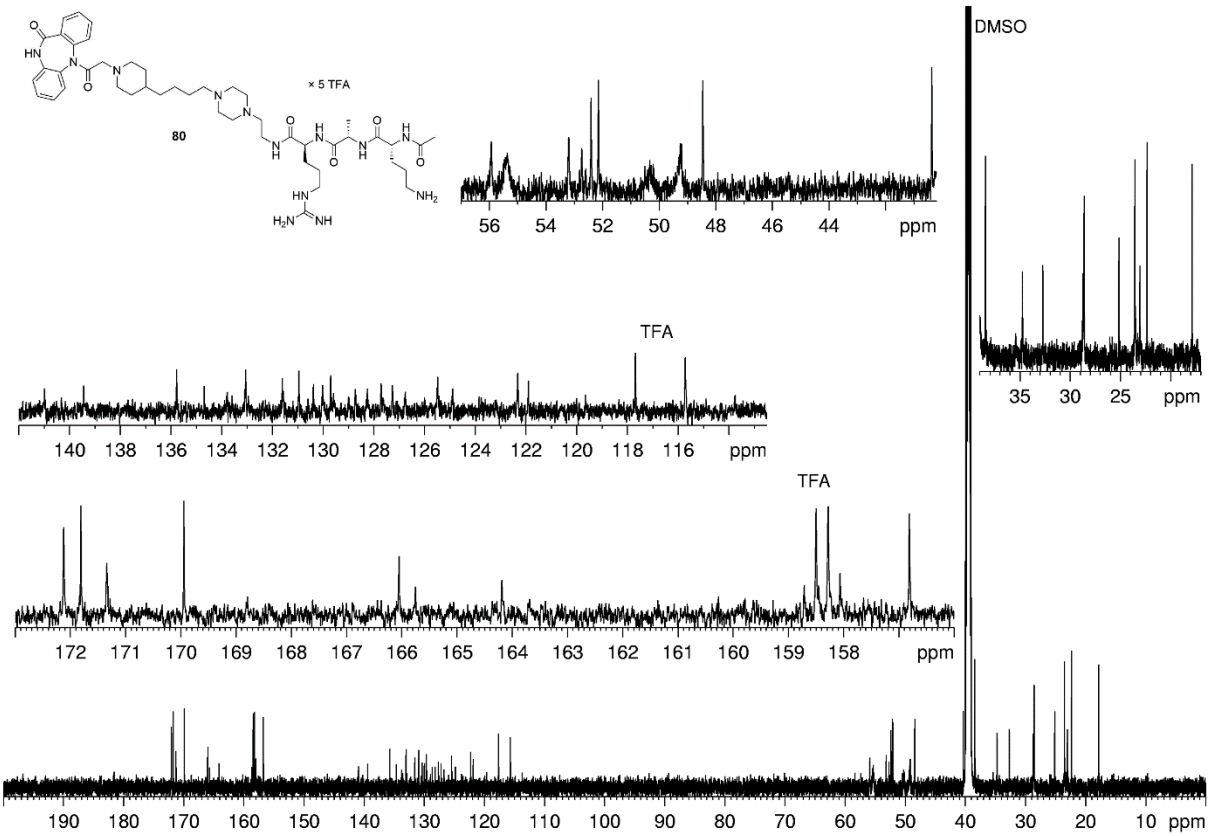
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **79**



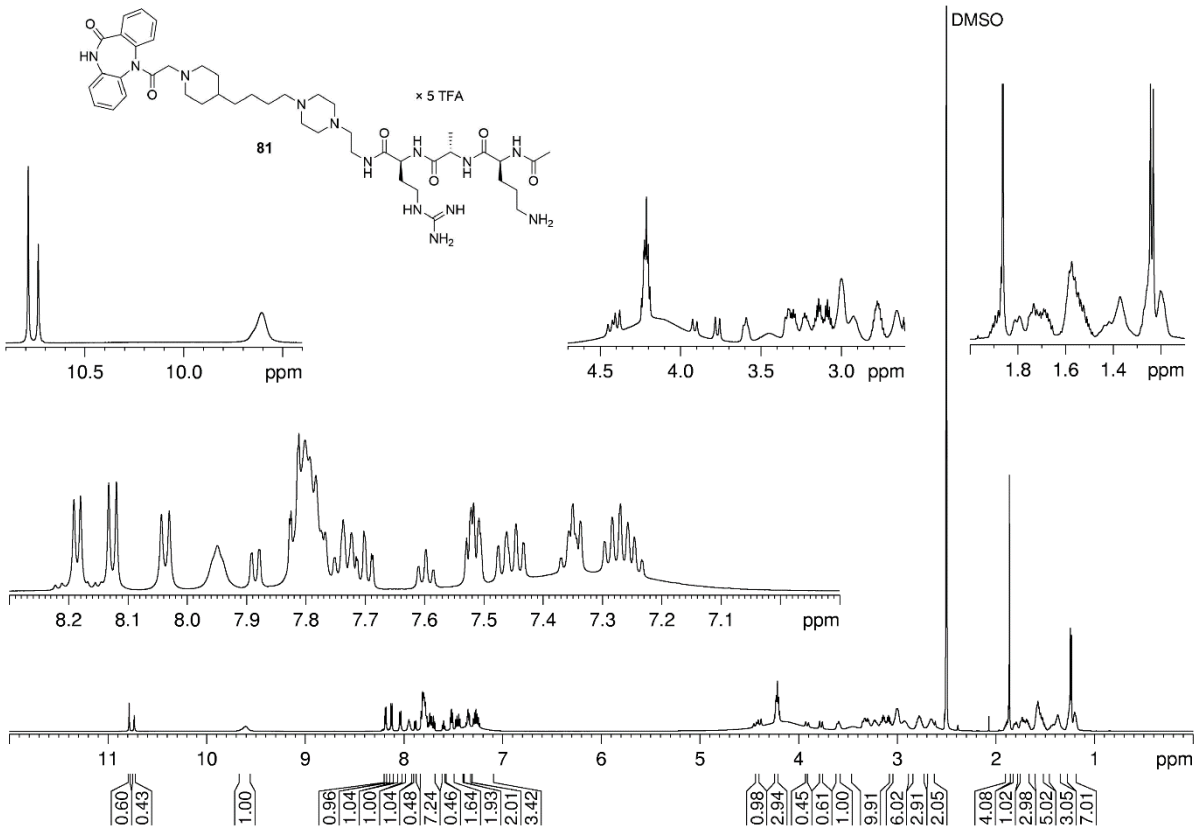
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **80**



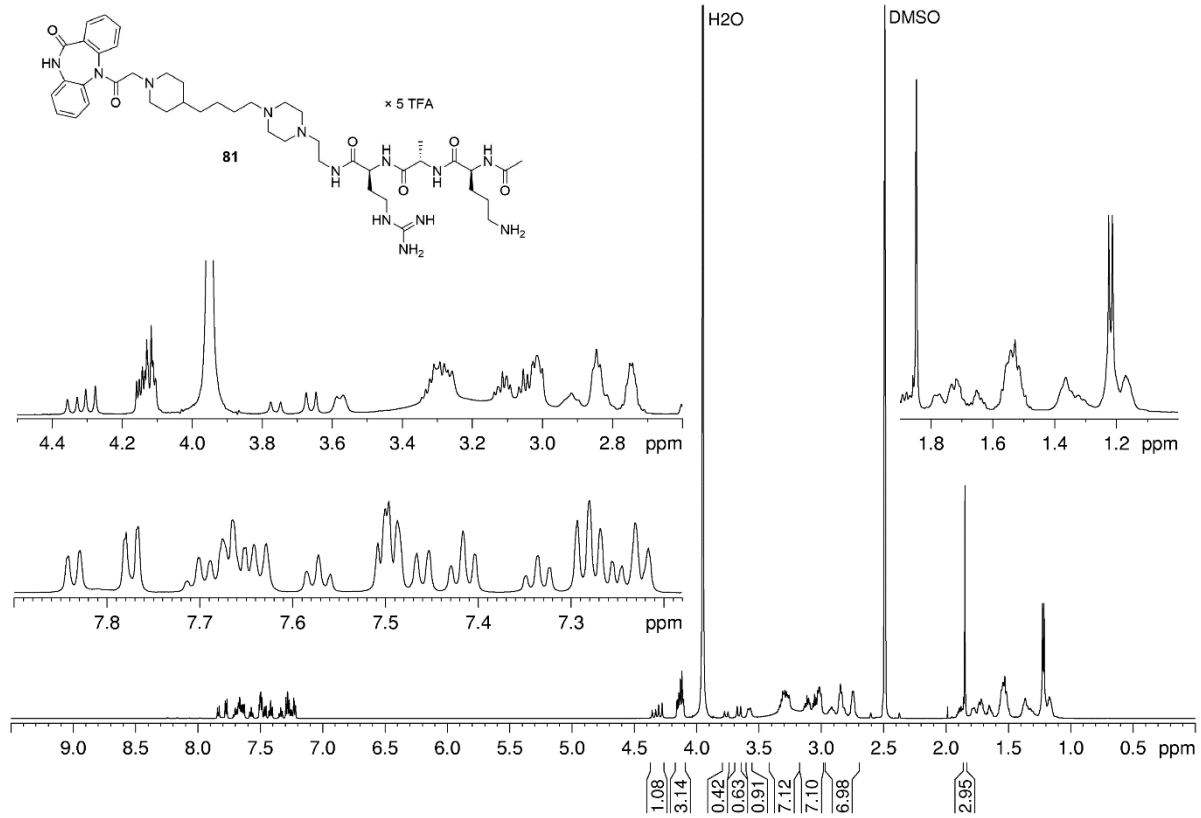
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **80**



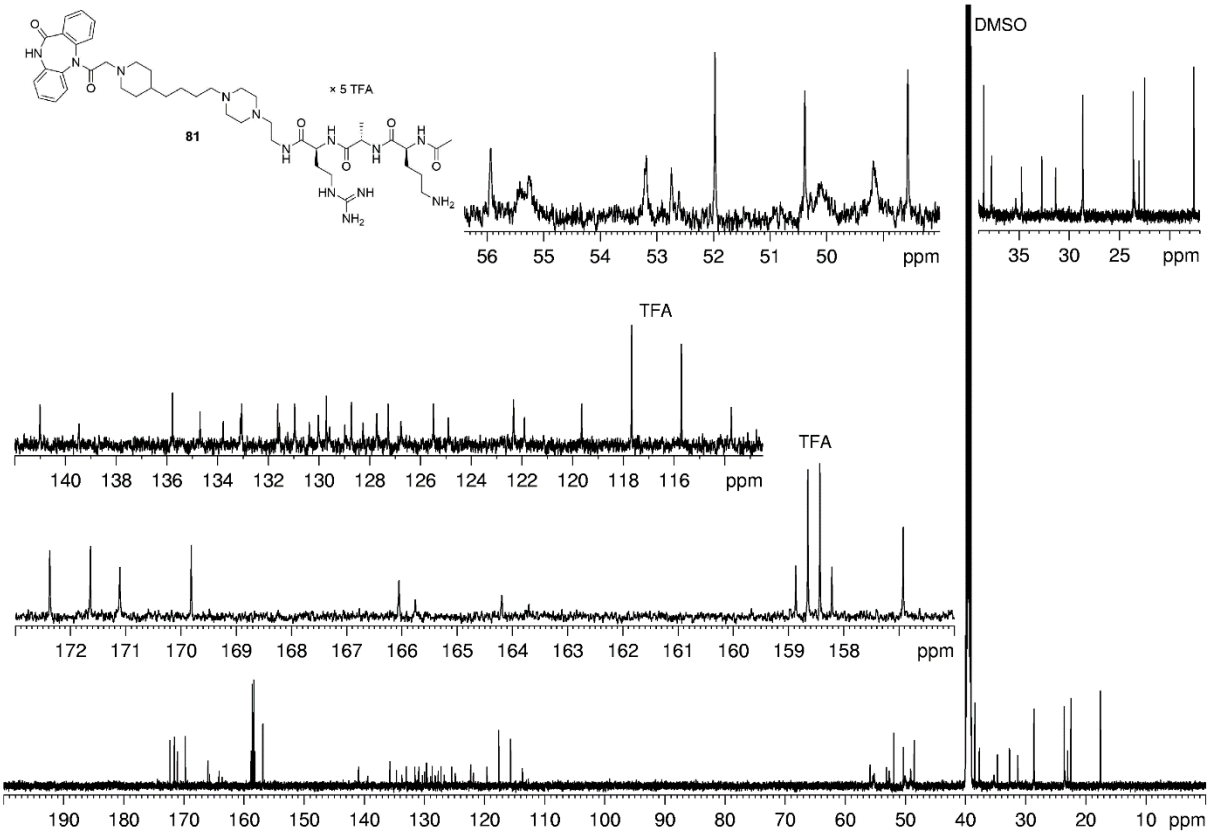
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **80**



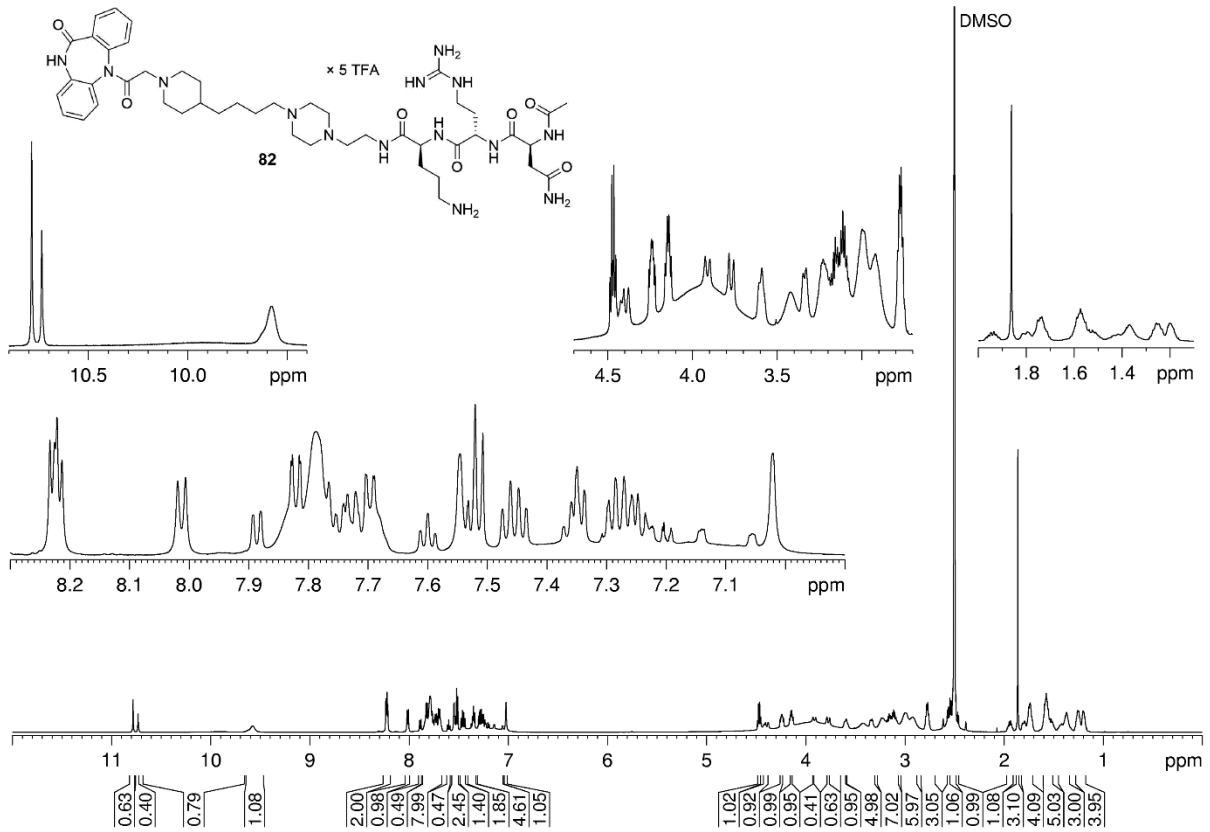
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **81**



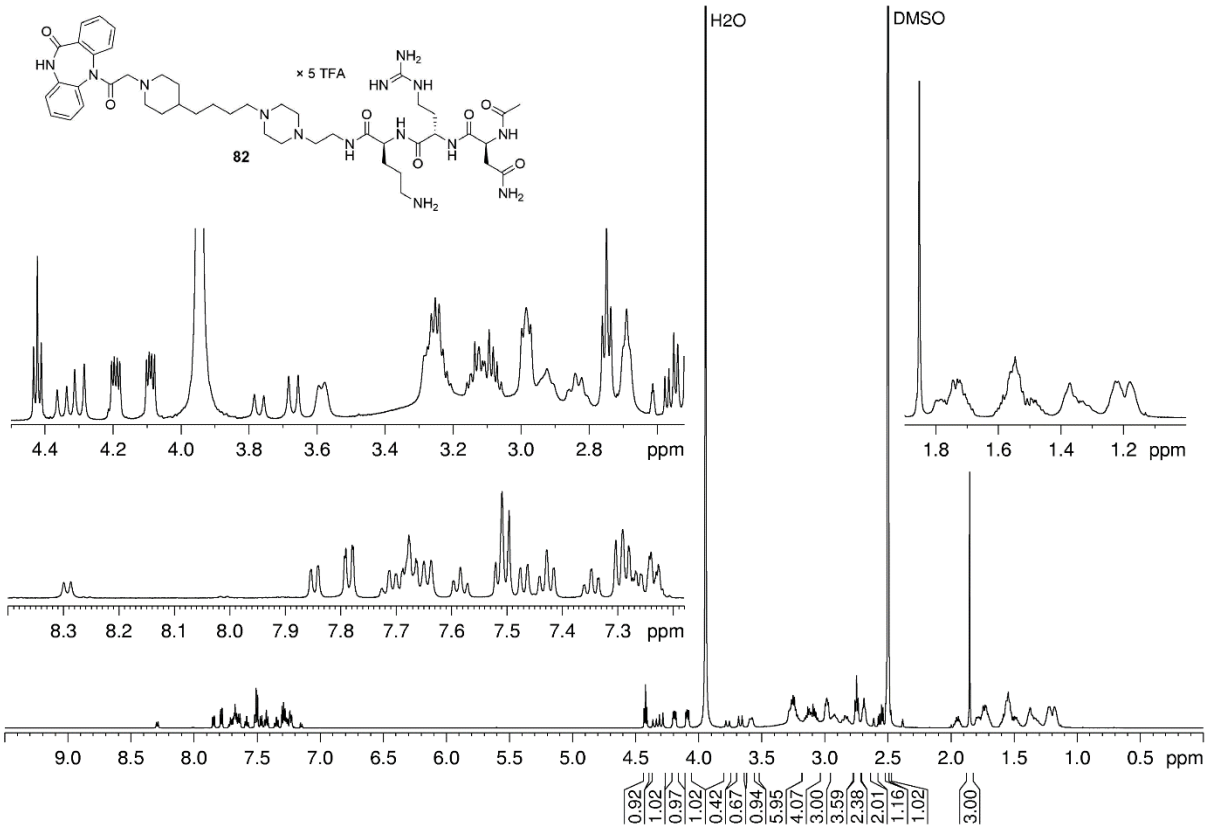
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **81**



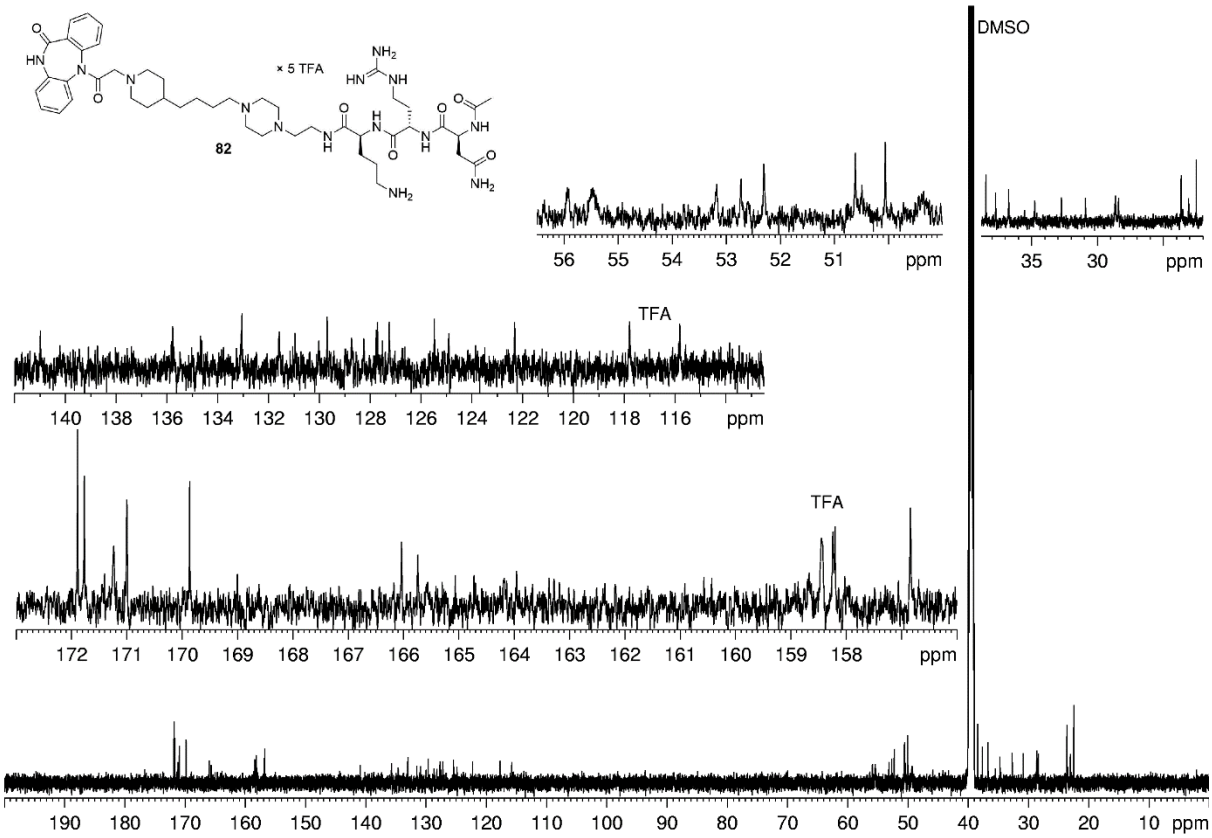
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **81**



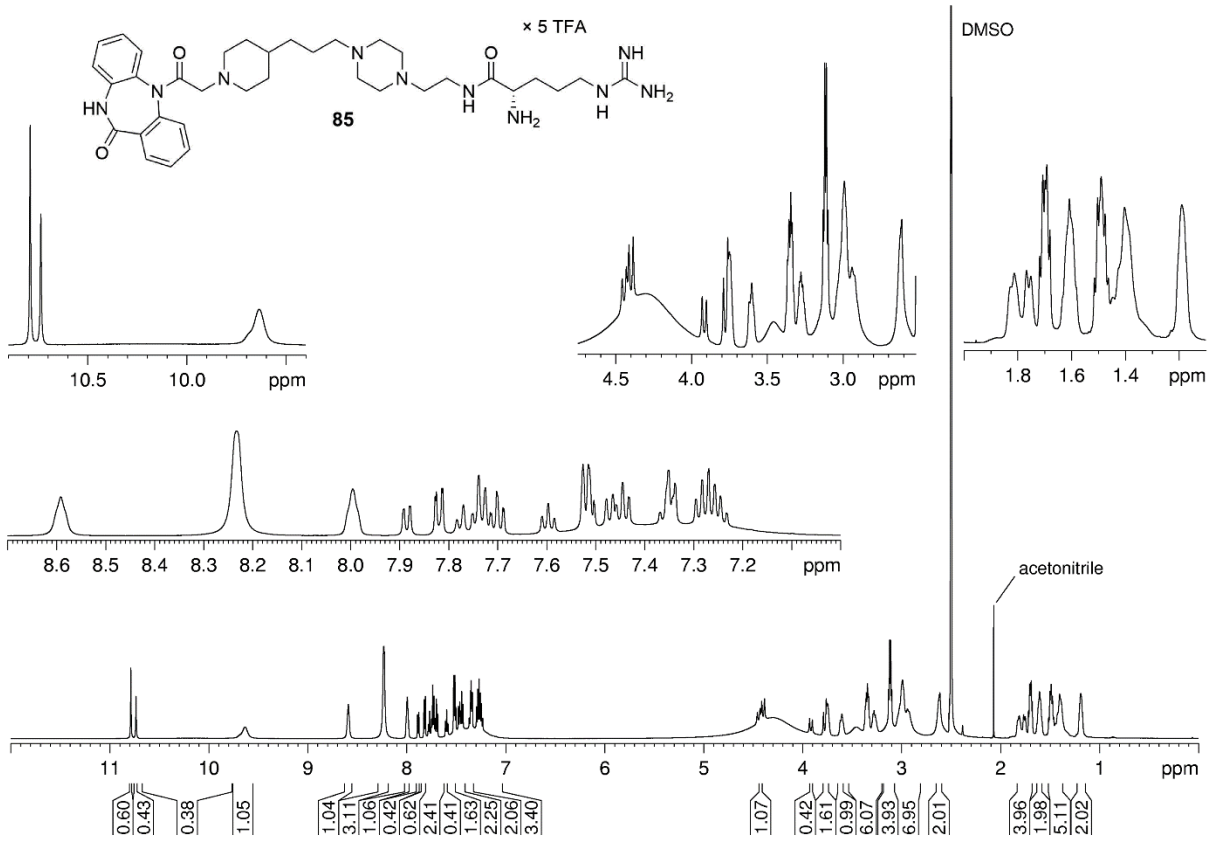
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **82**



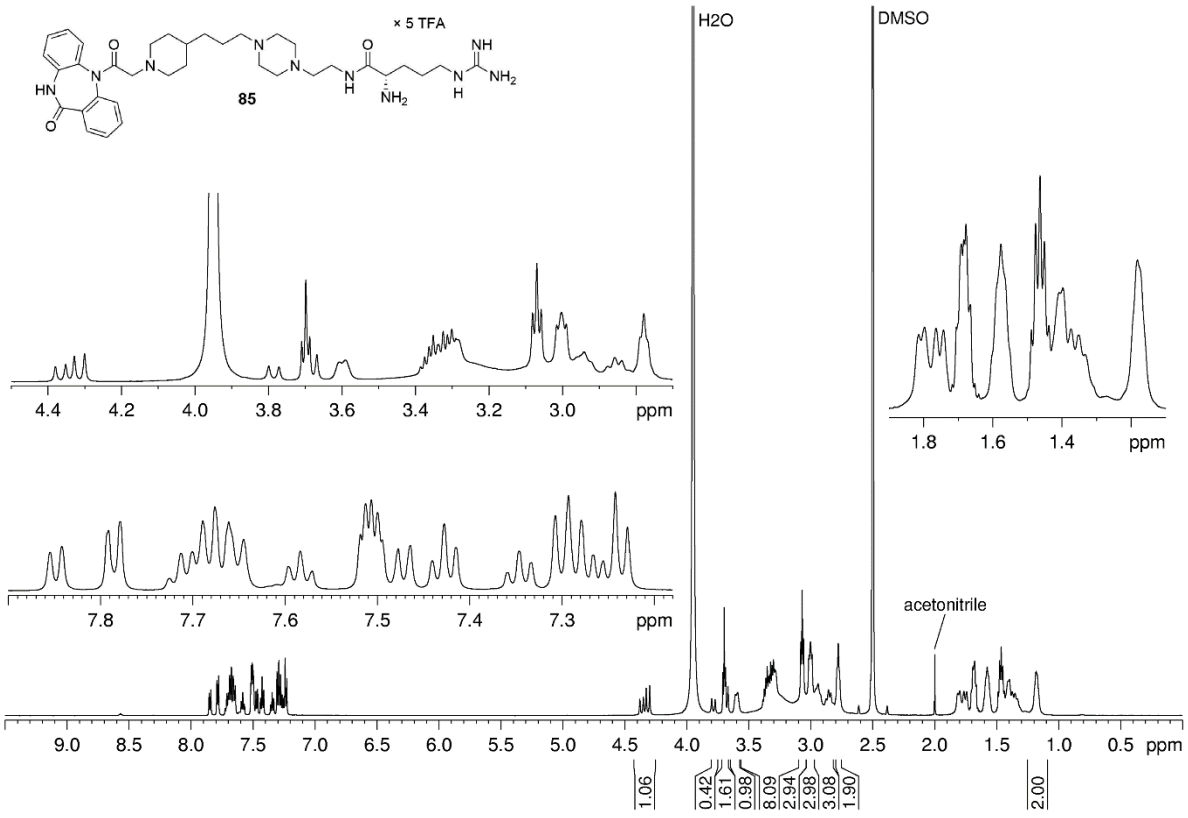
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **82**



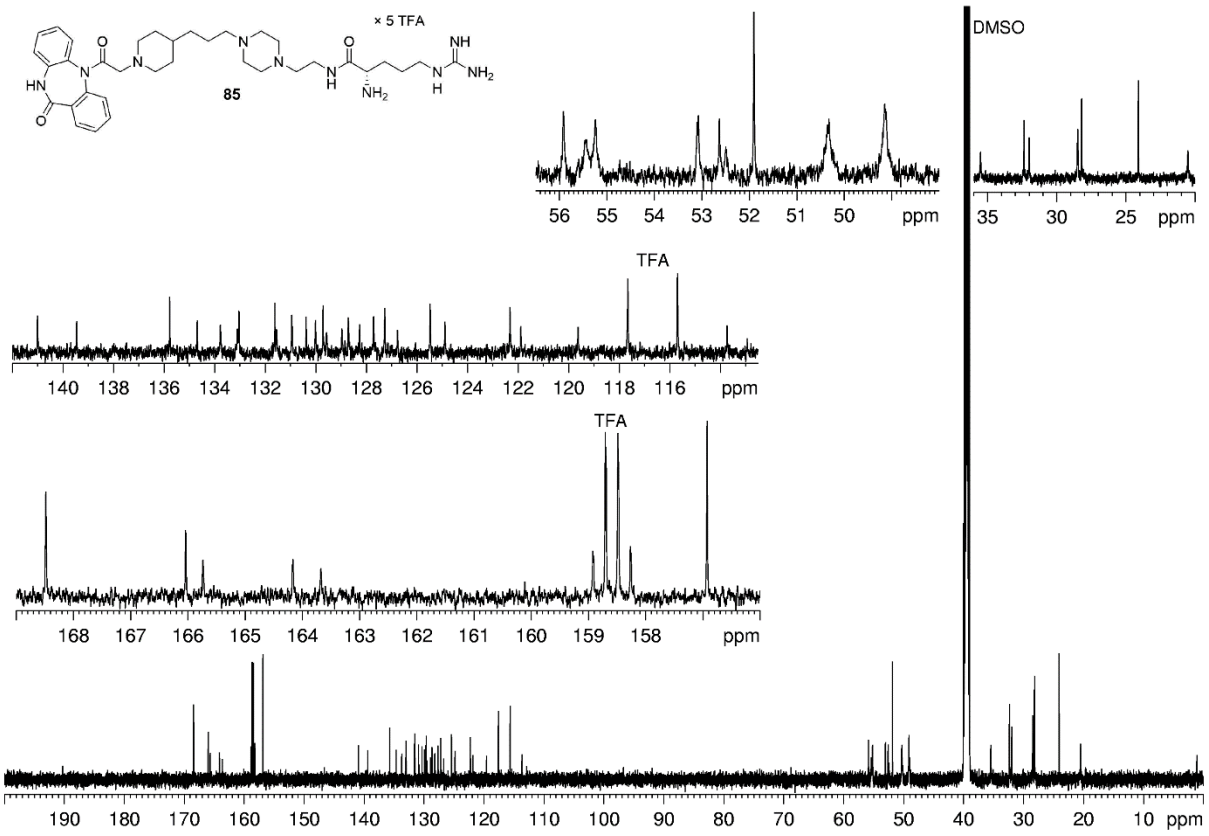
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **82**



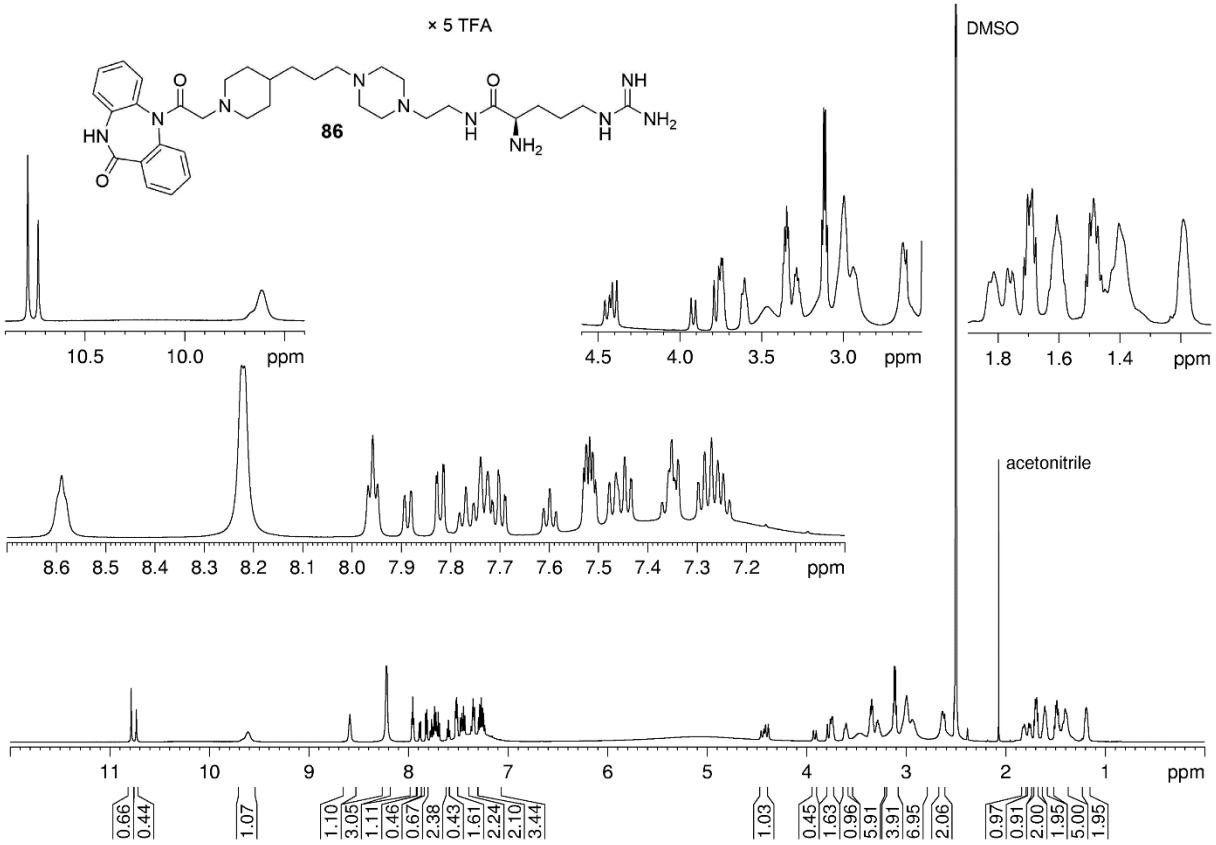
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **85**



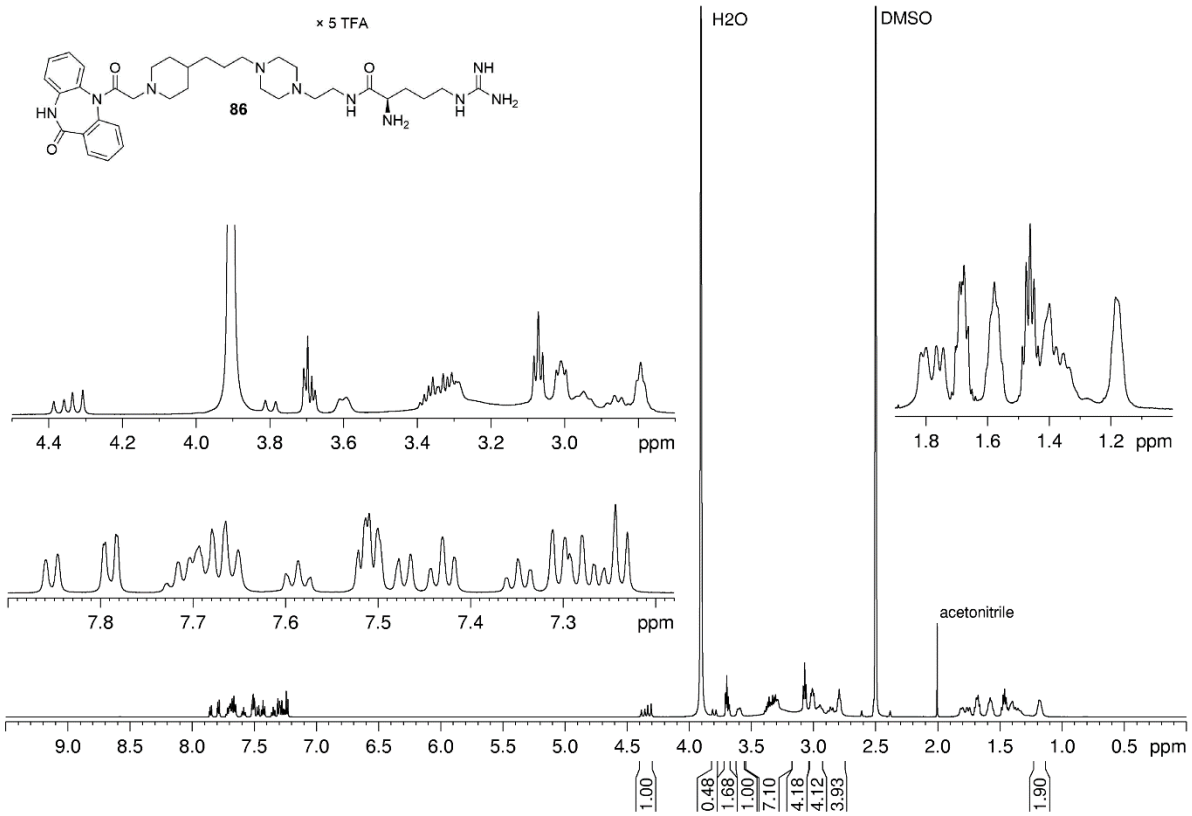
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **85**



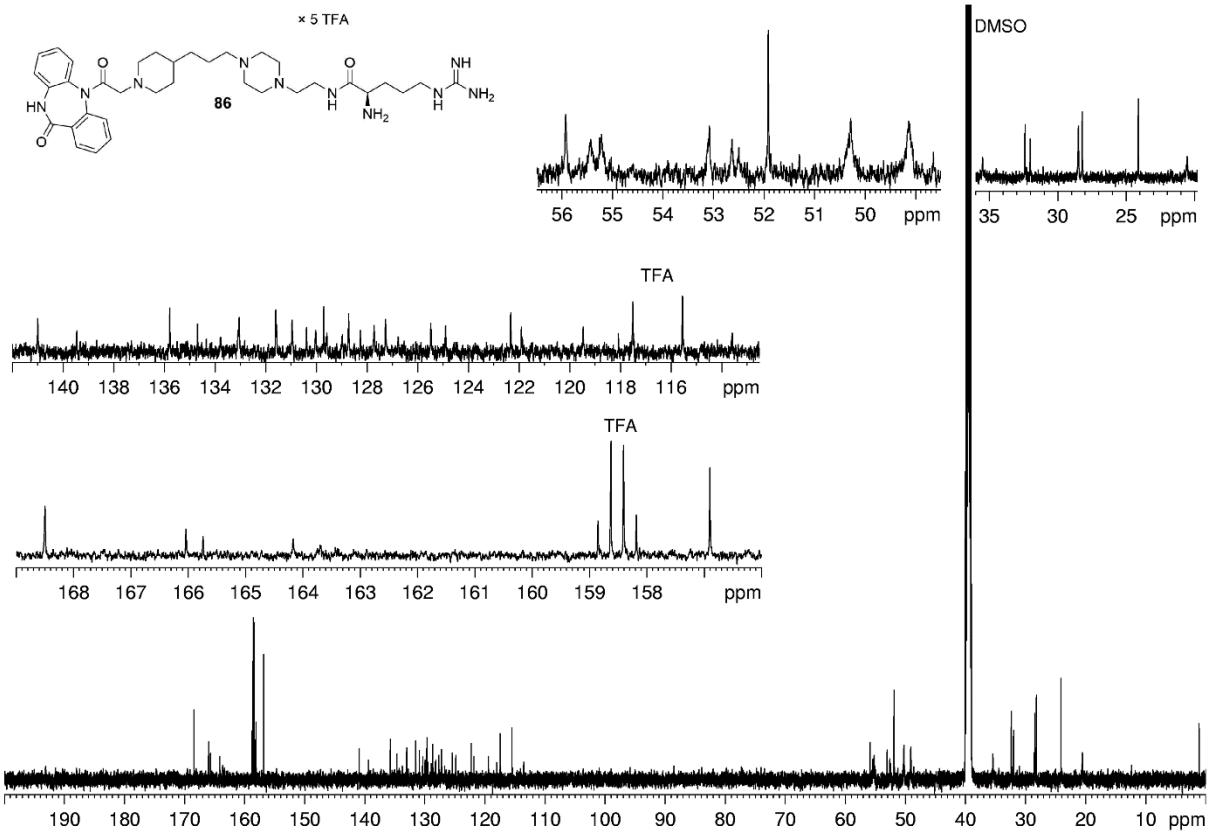
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **85**



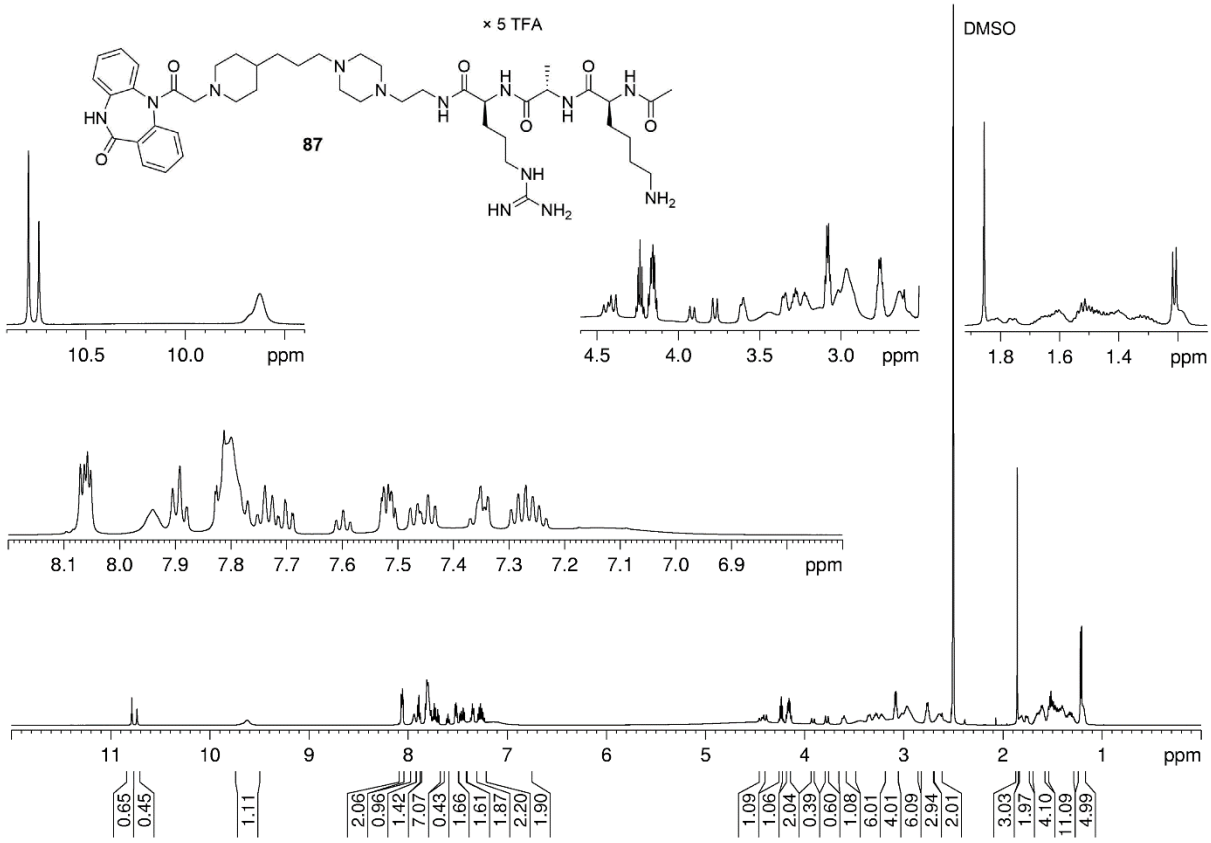
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **86**



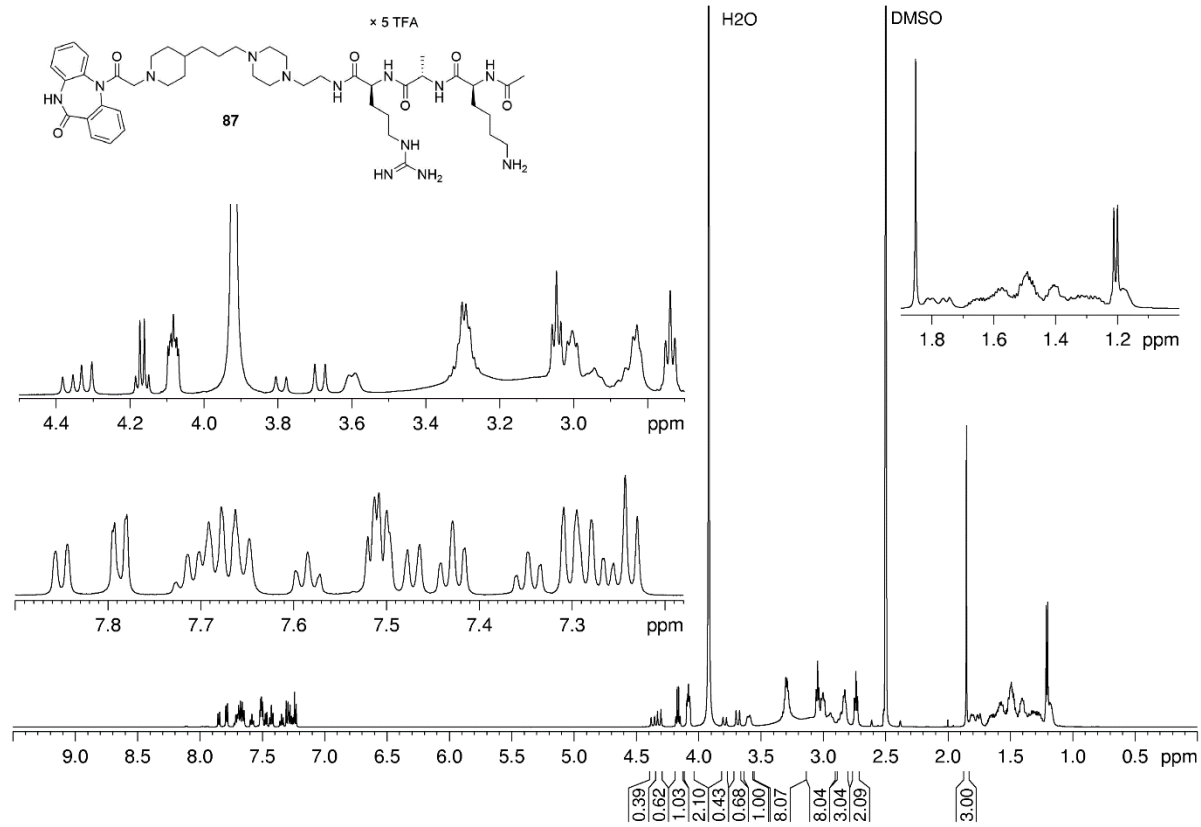
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **86**



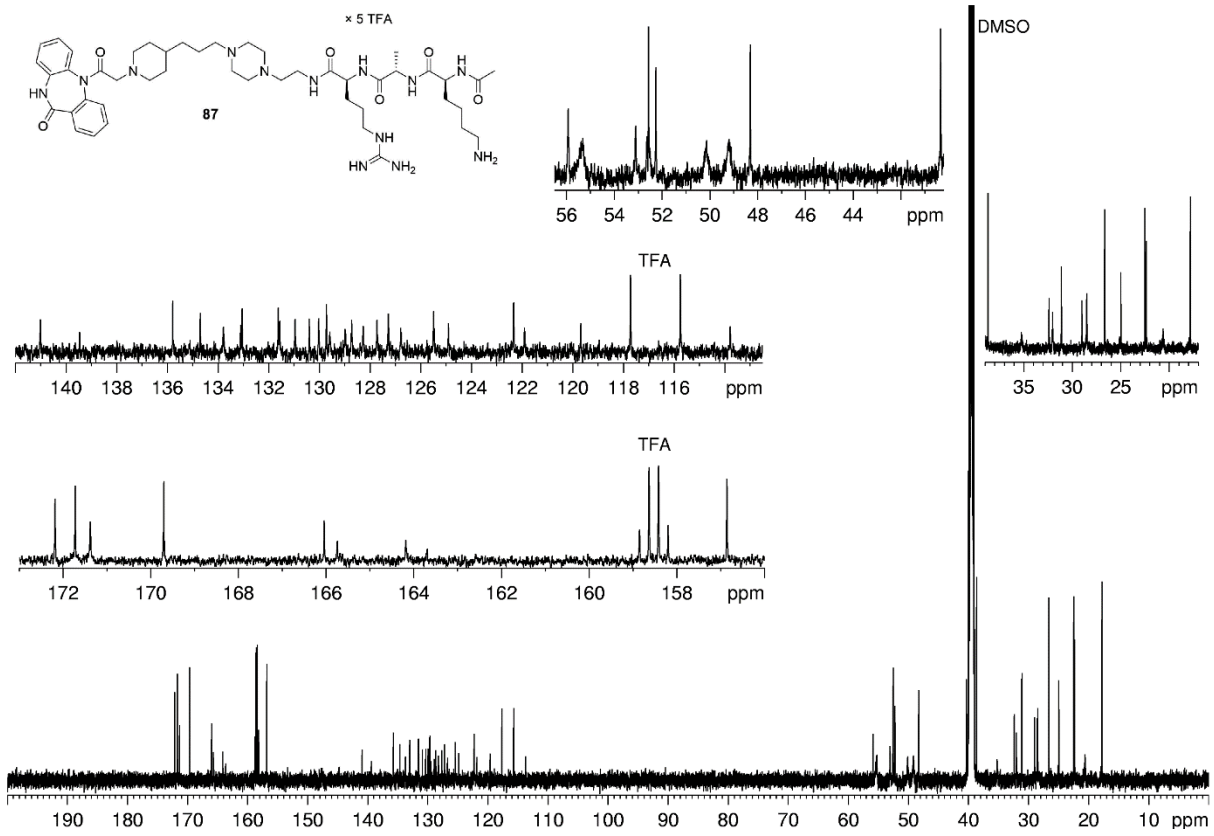
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **86**



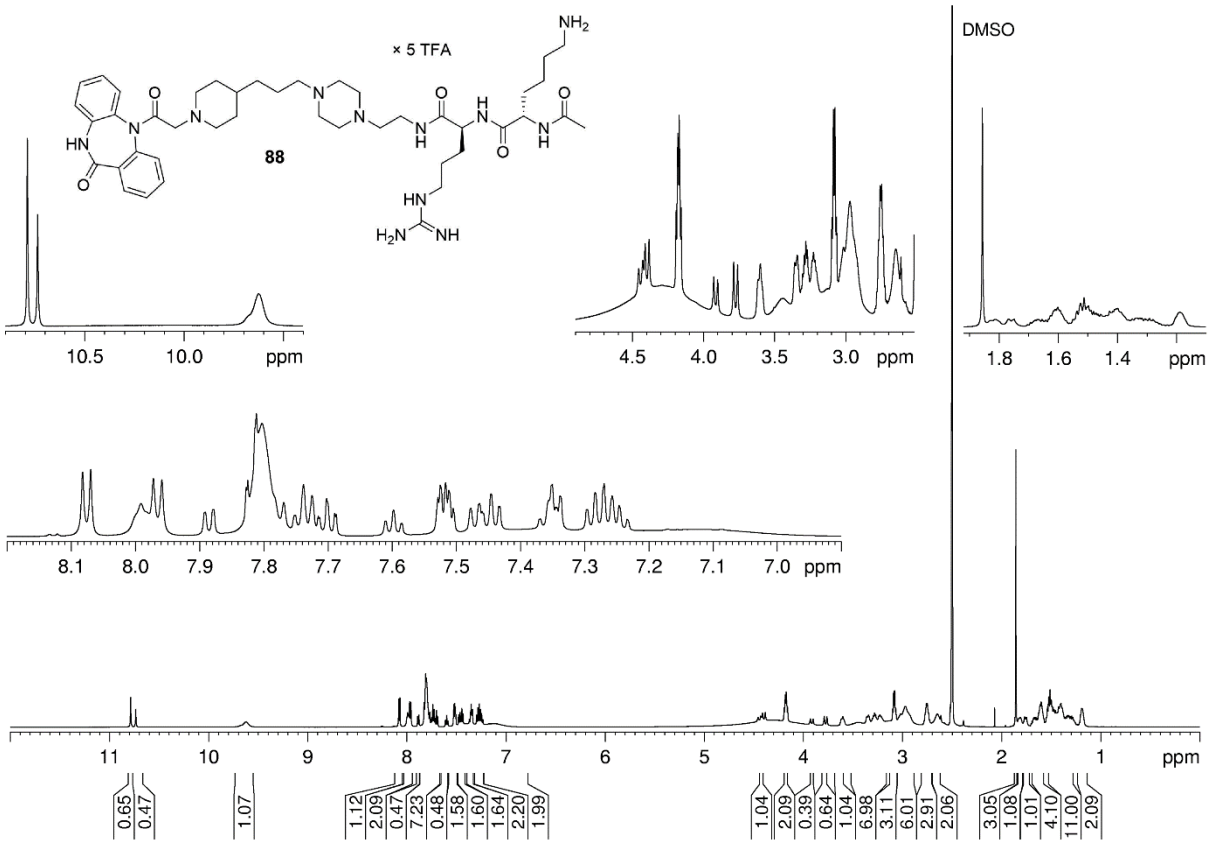
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **87**



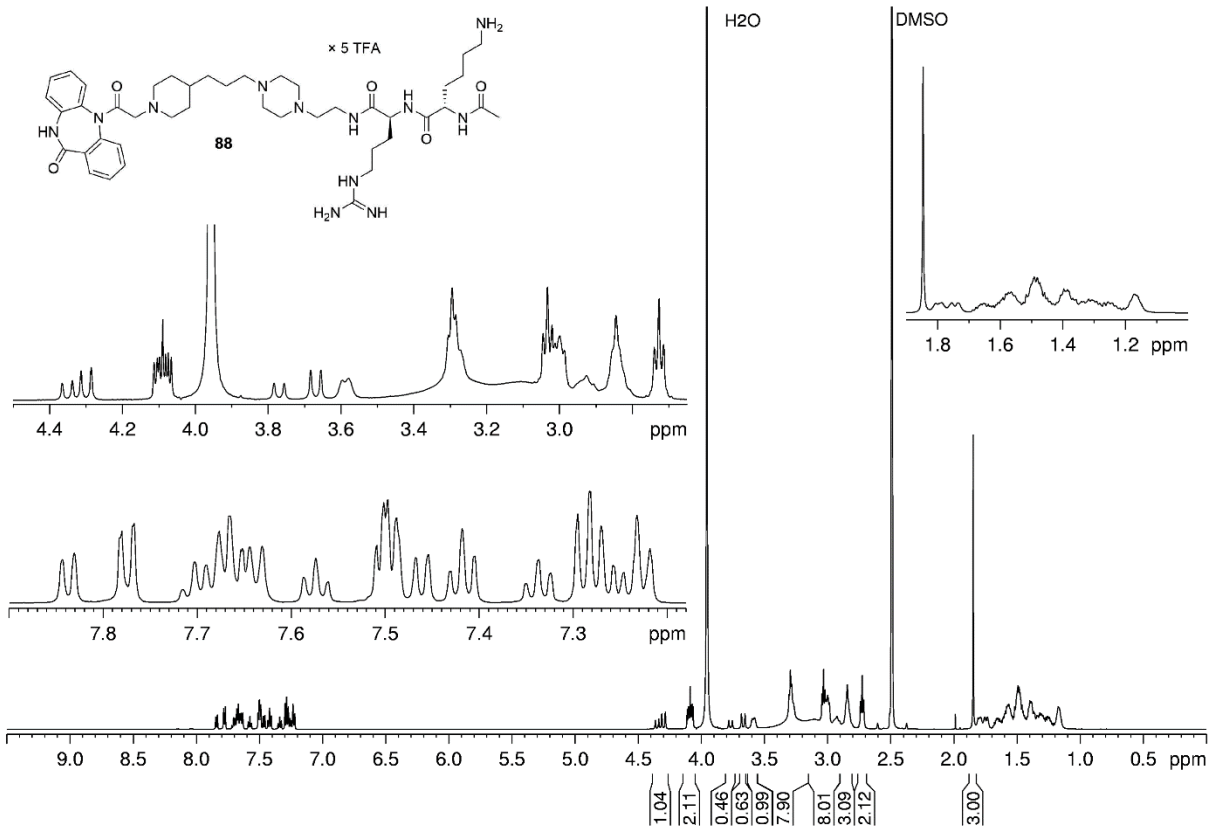
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **87**



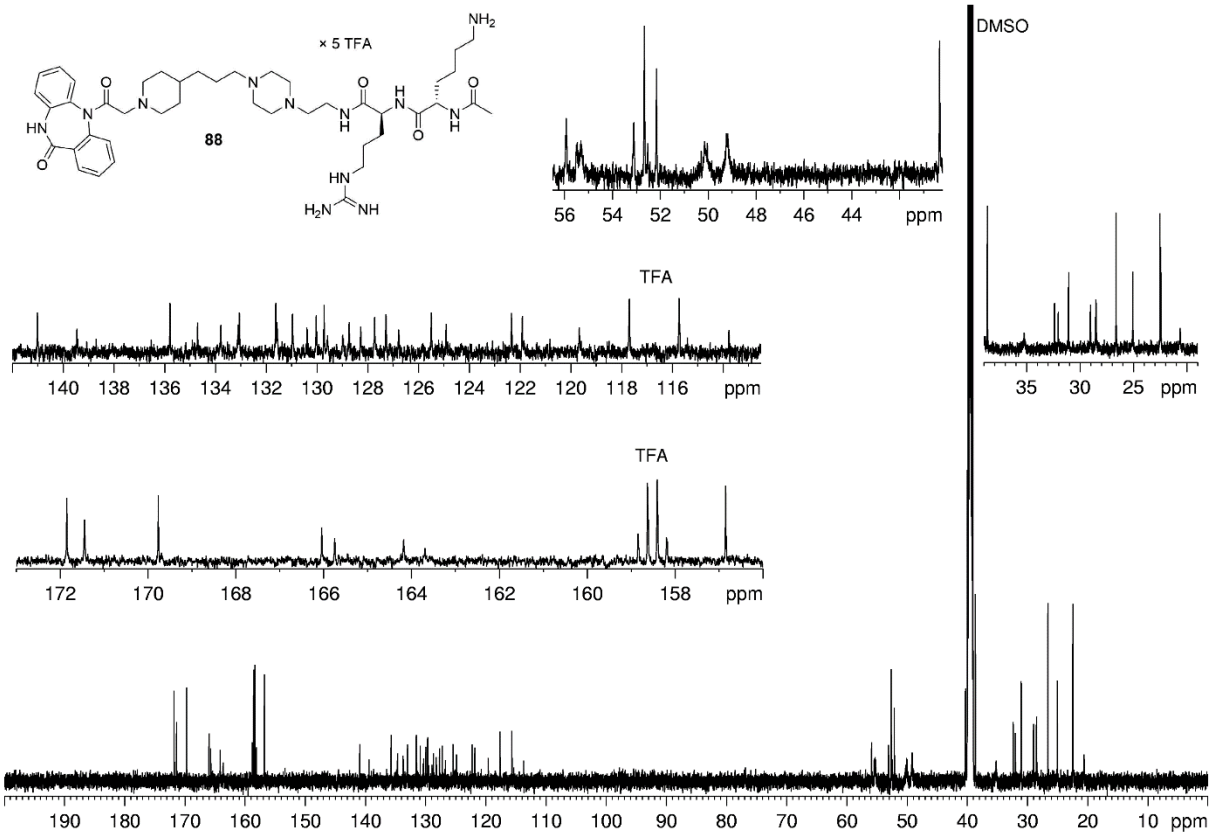
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **87**



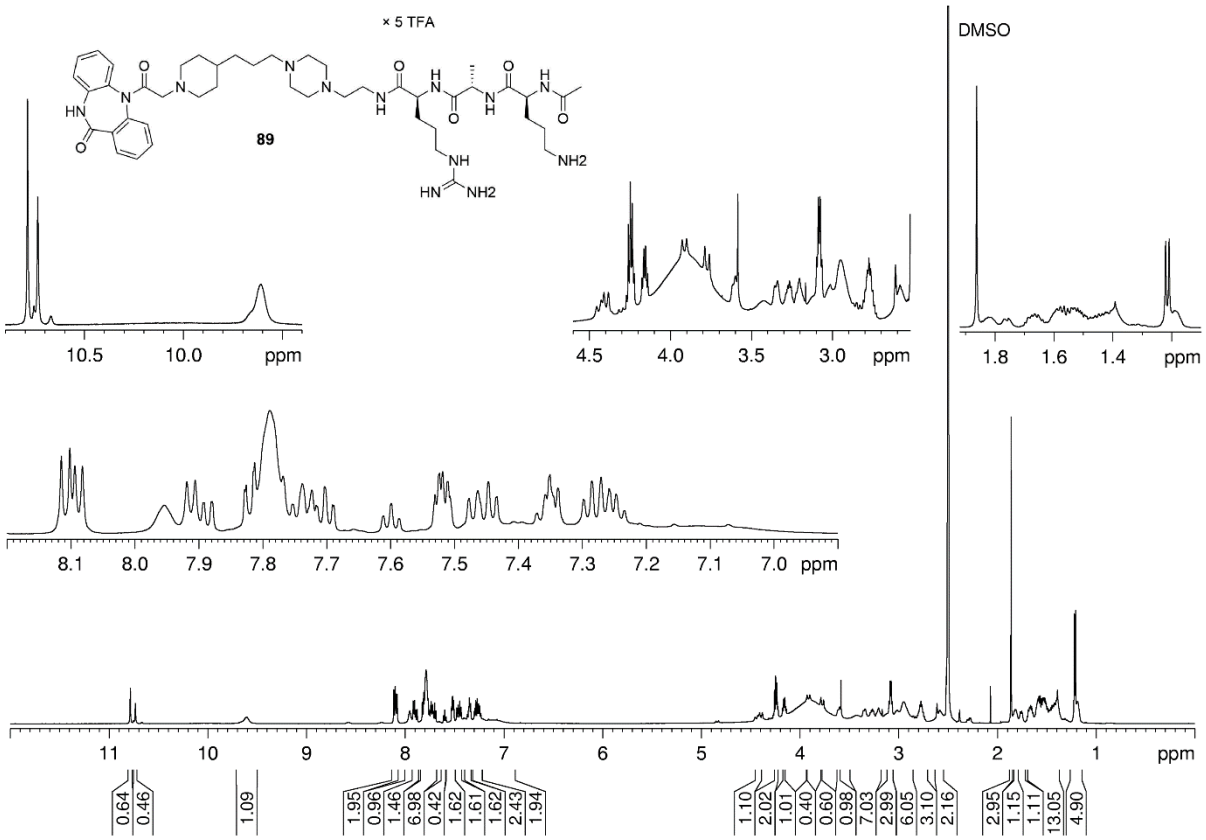
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **88**



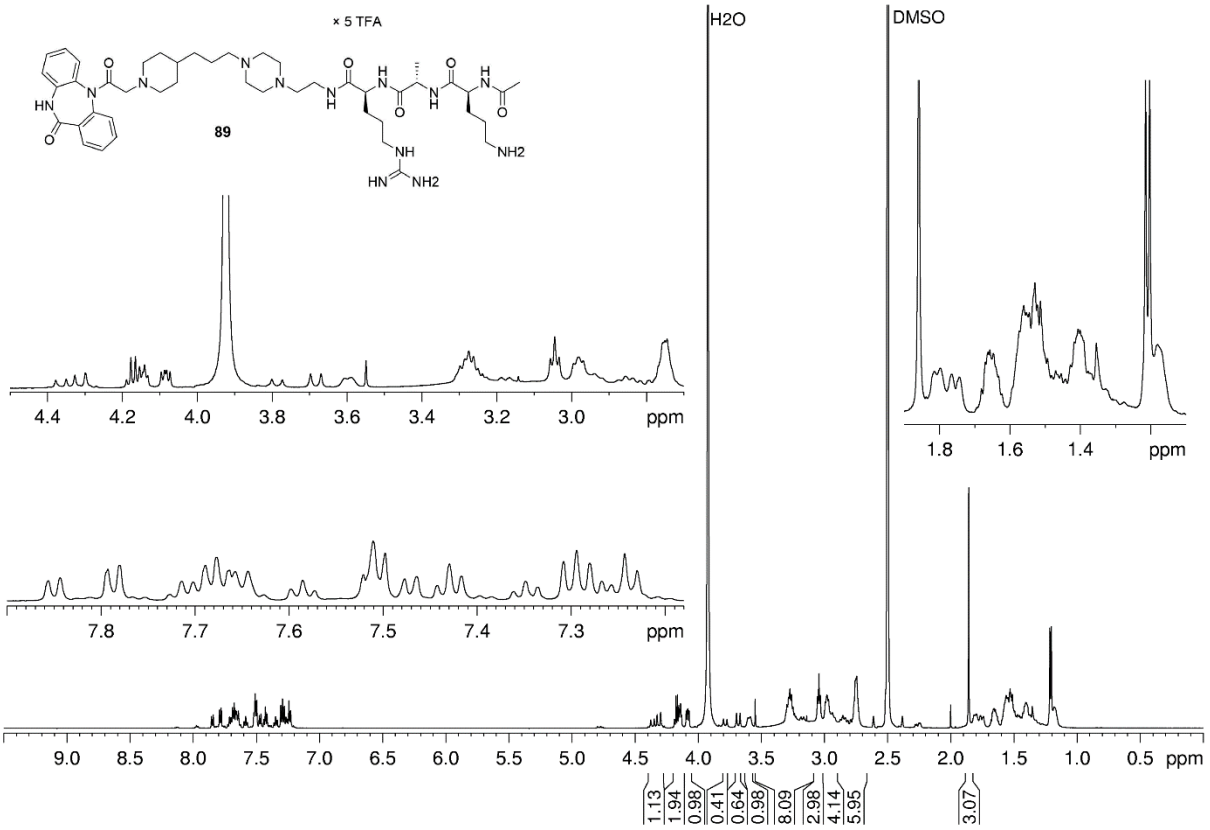
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **88**



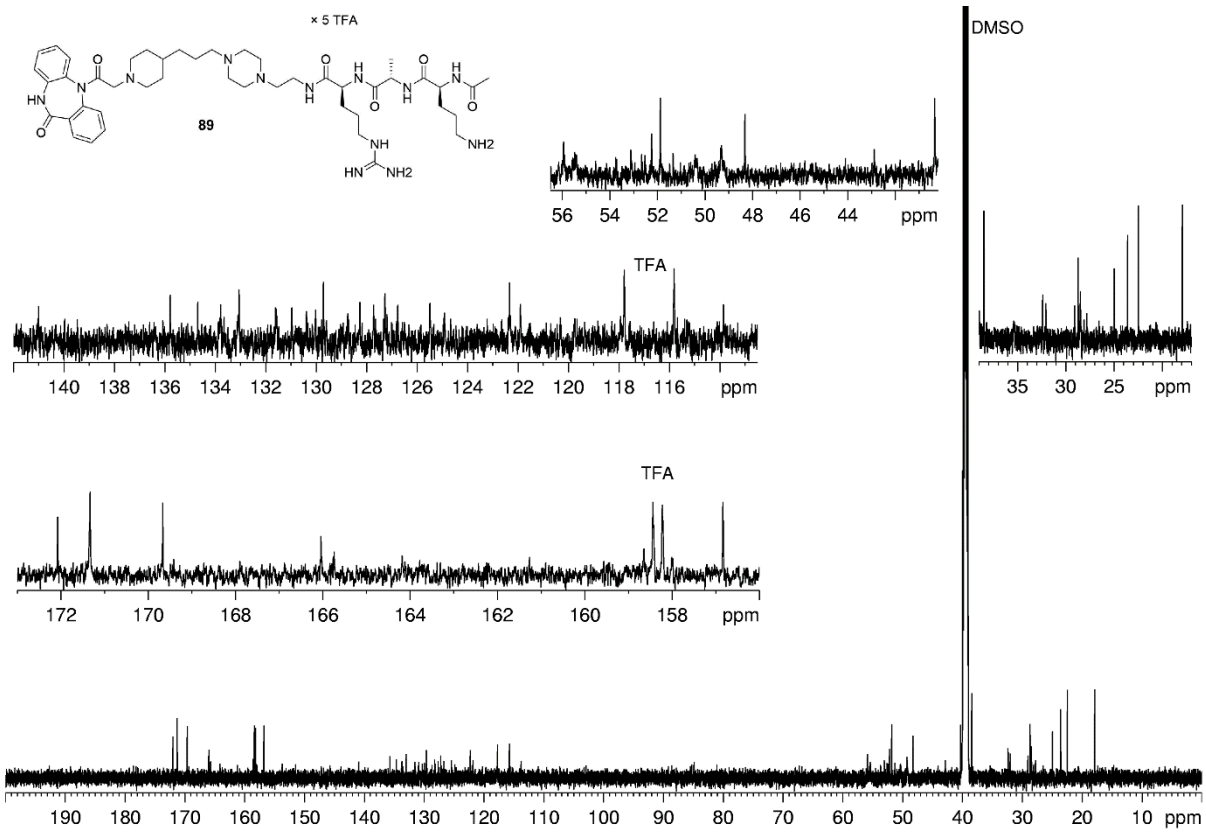
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **88**



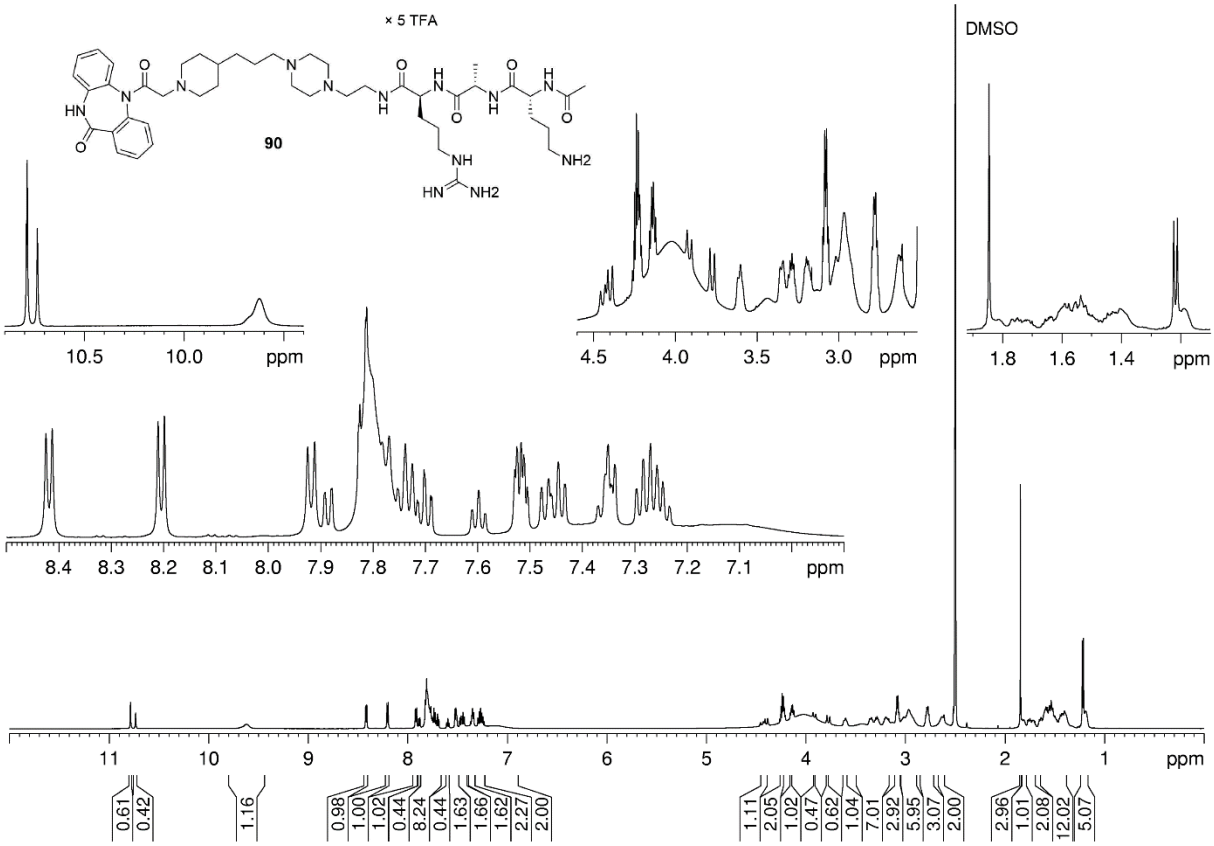
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **89**



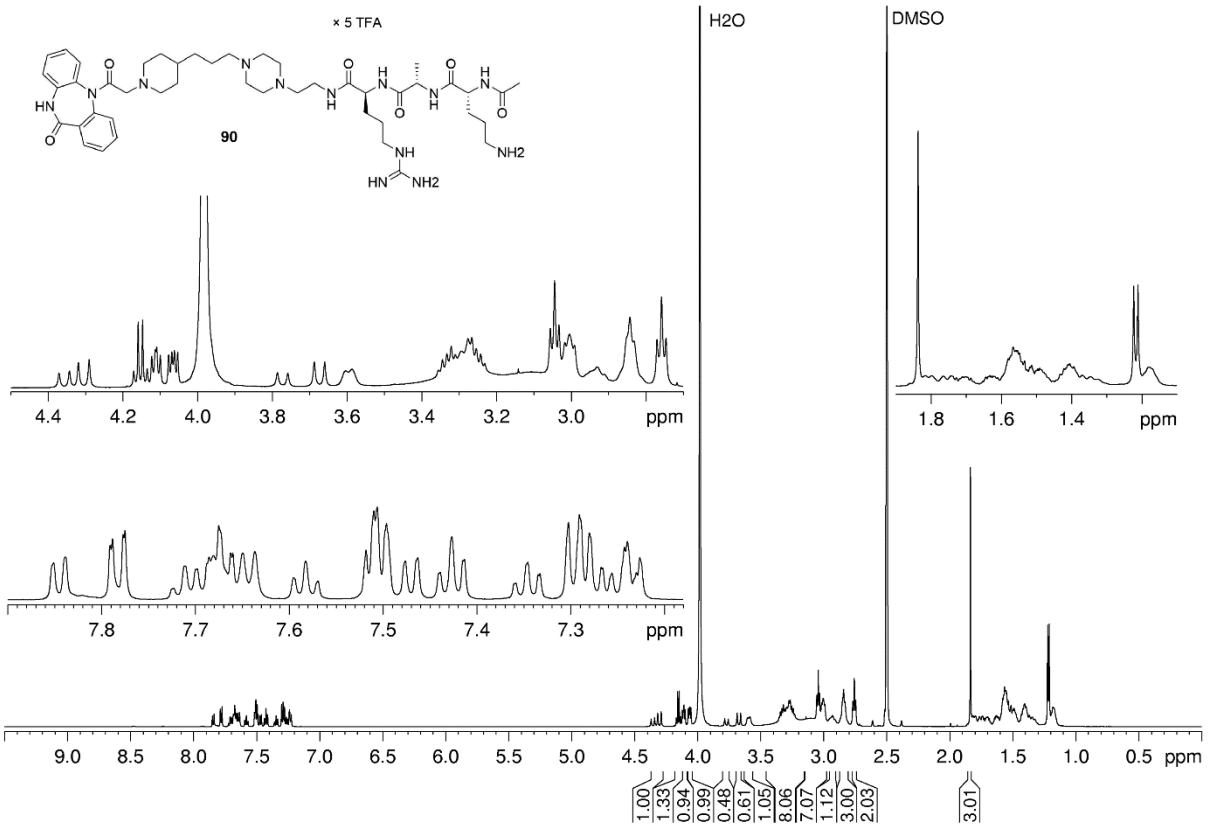
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **89**



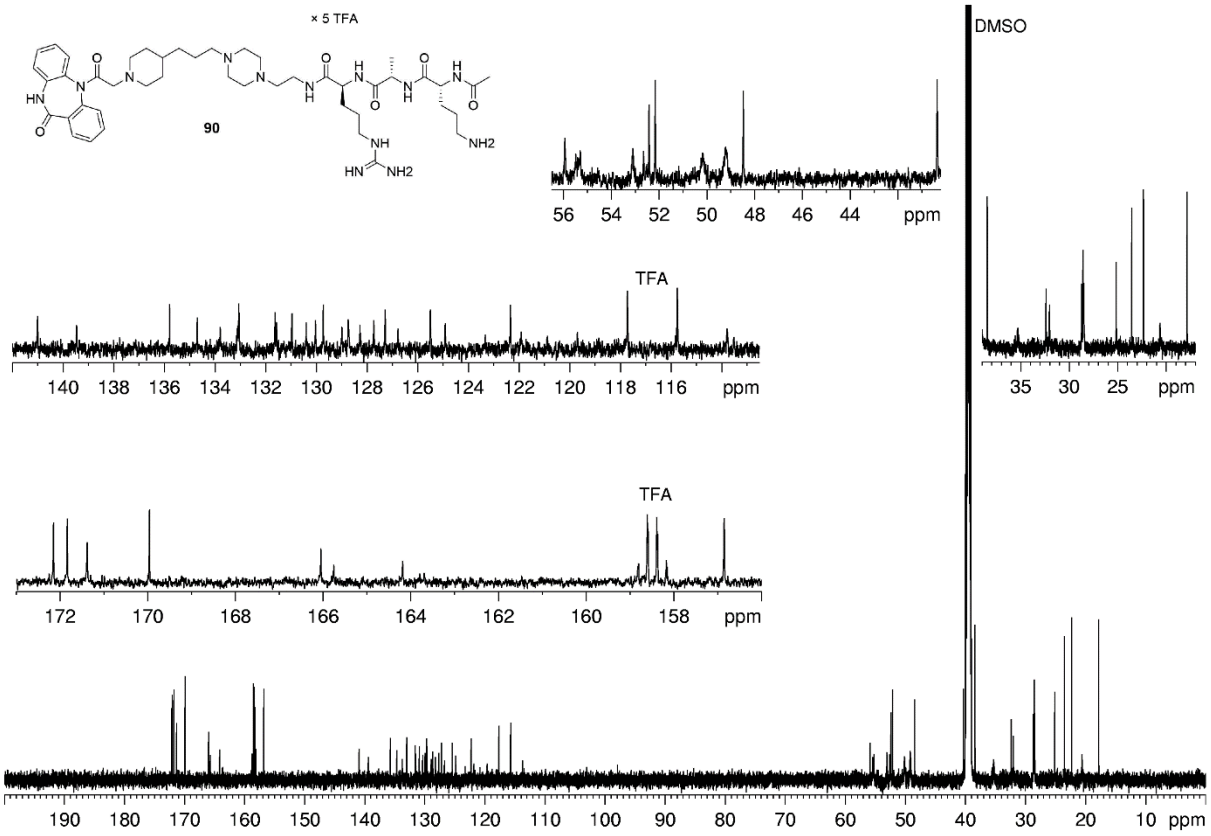
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **89**



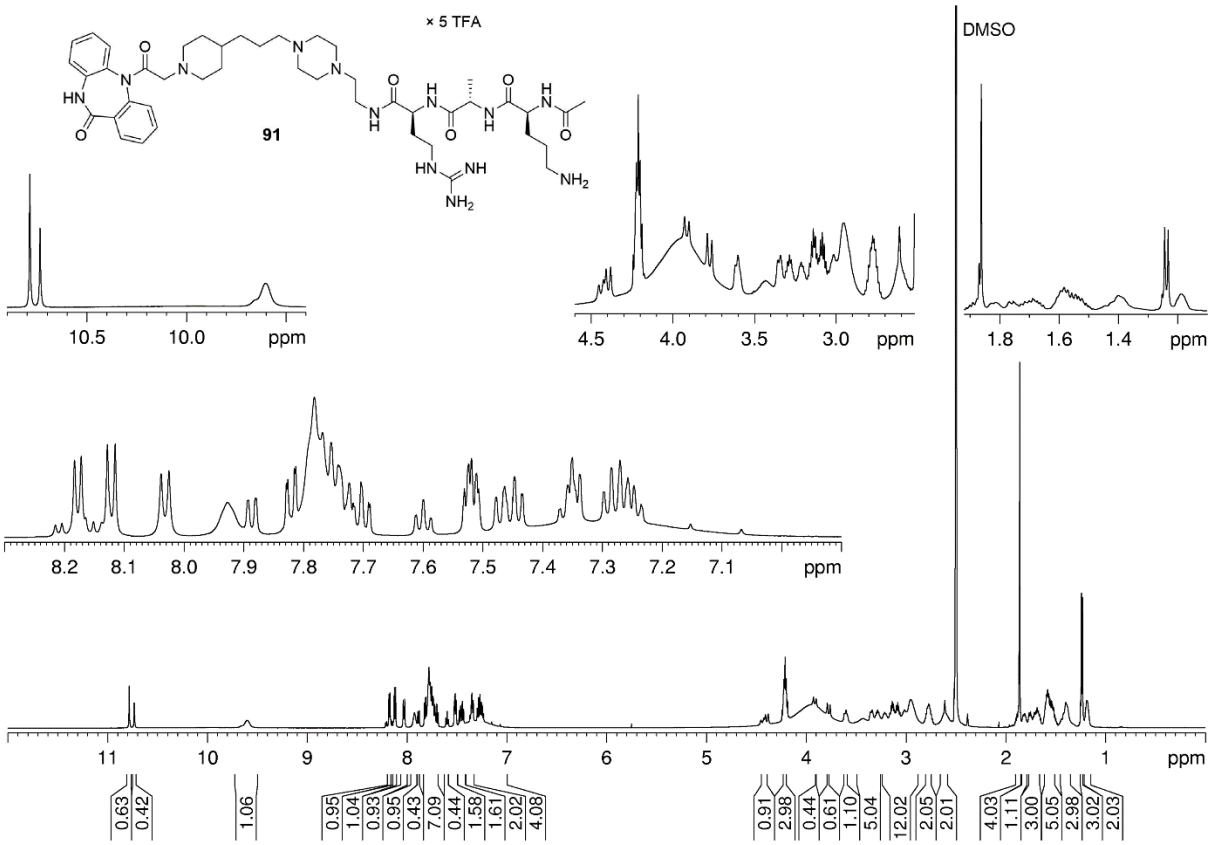
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **90**



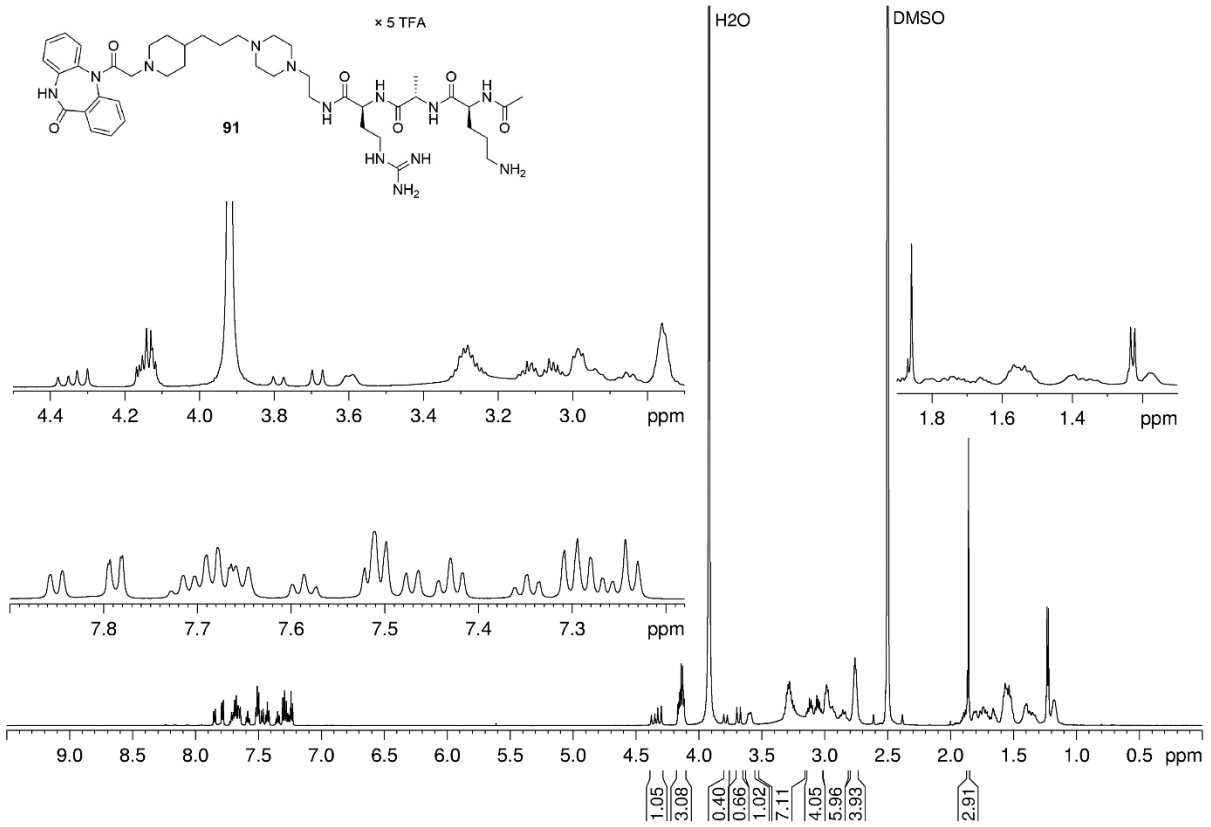
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **90**



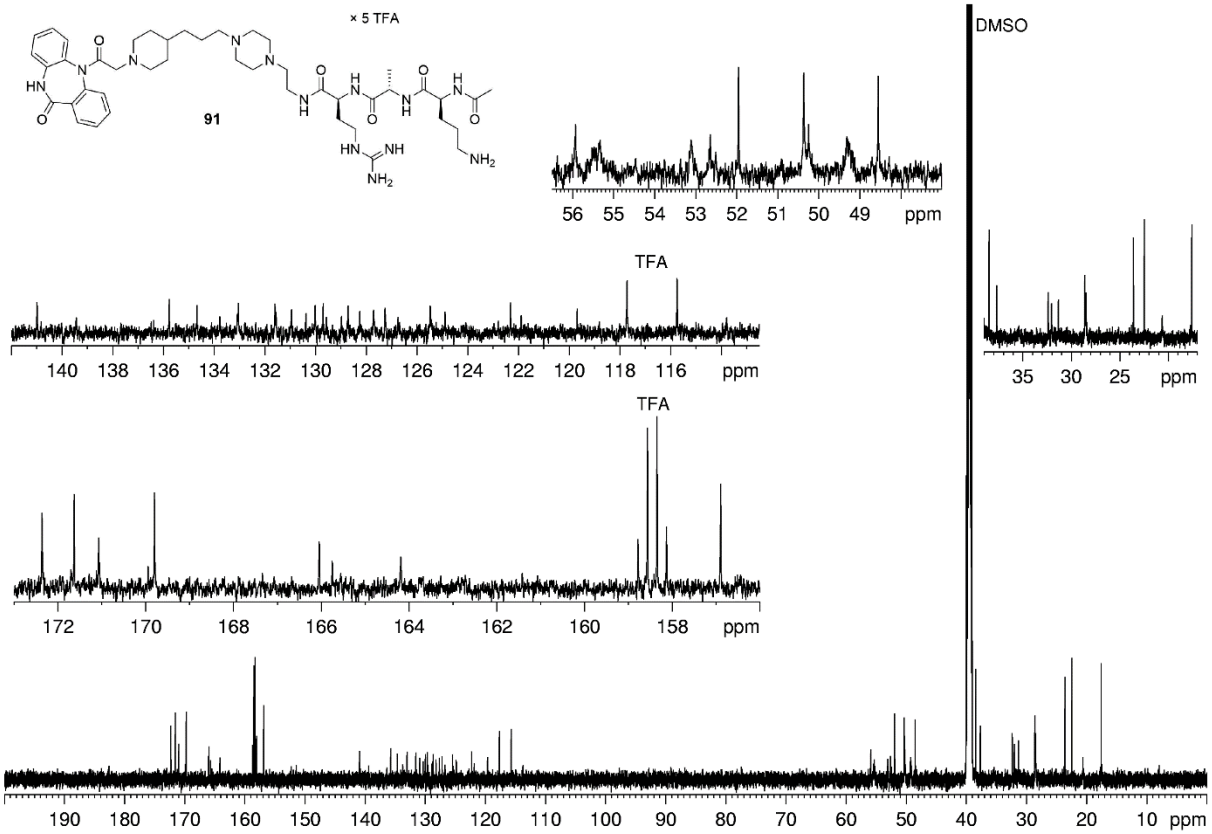
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **90**



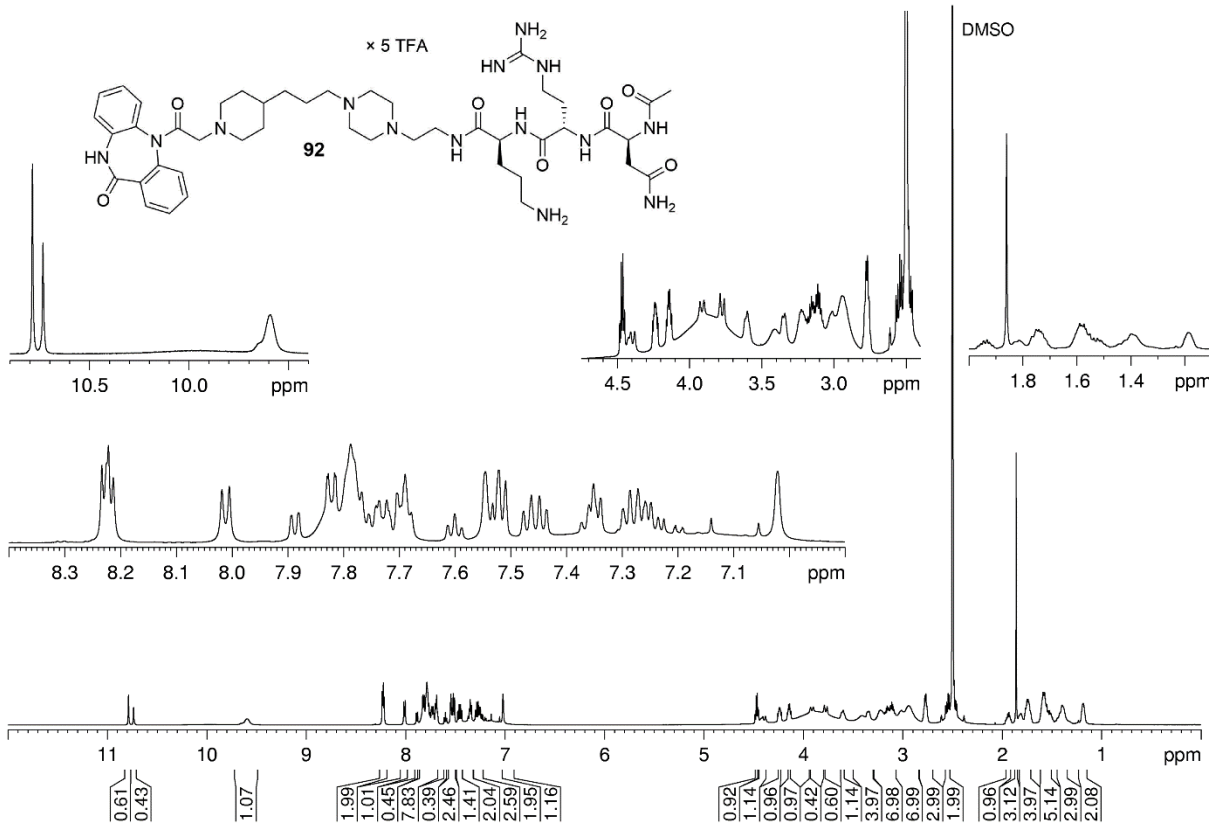
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **91**



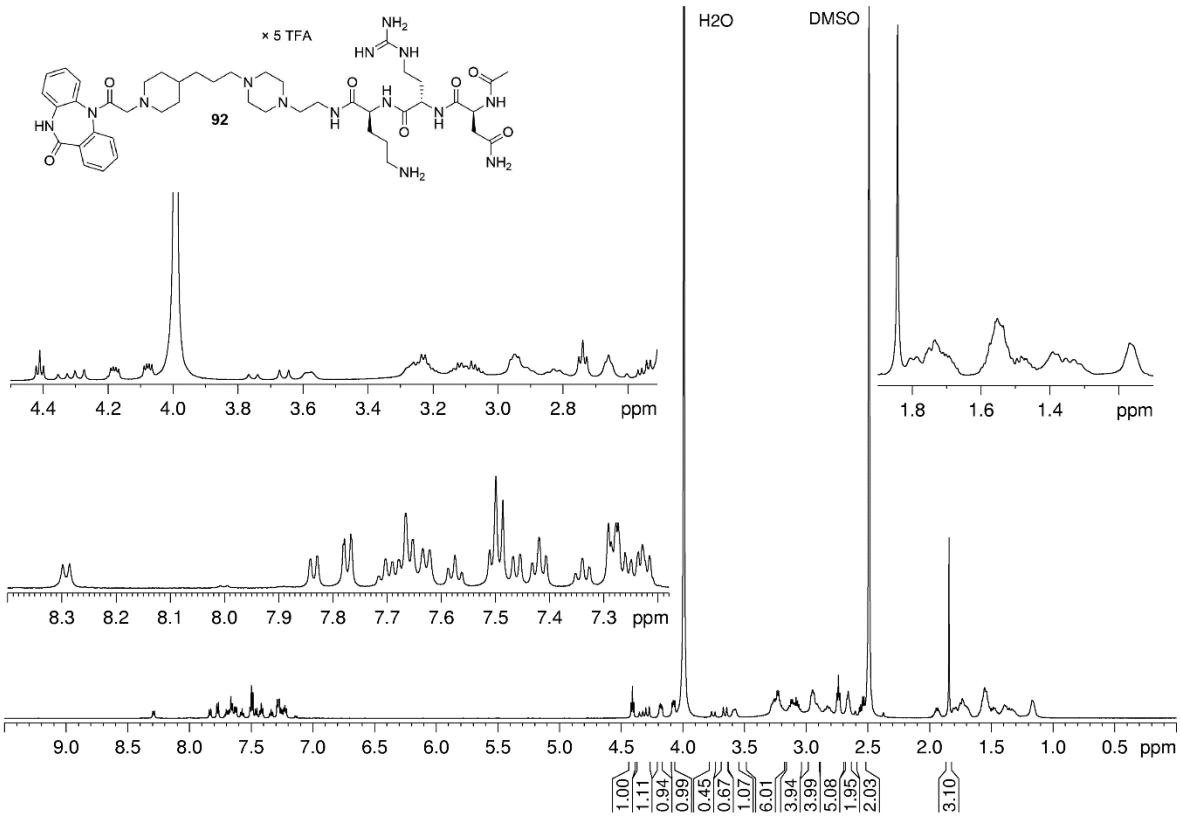
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **91**



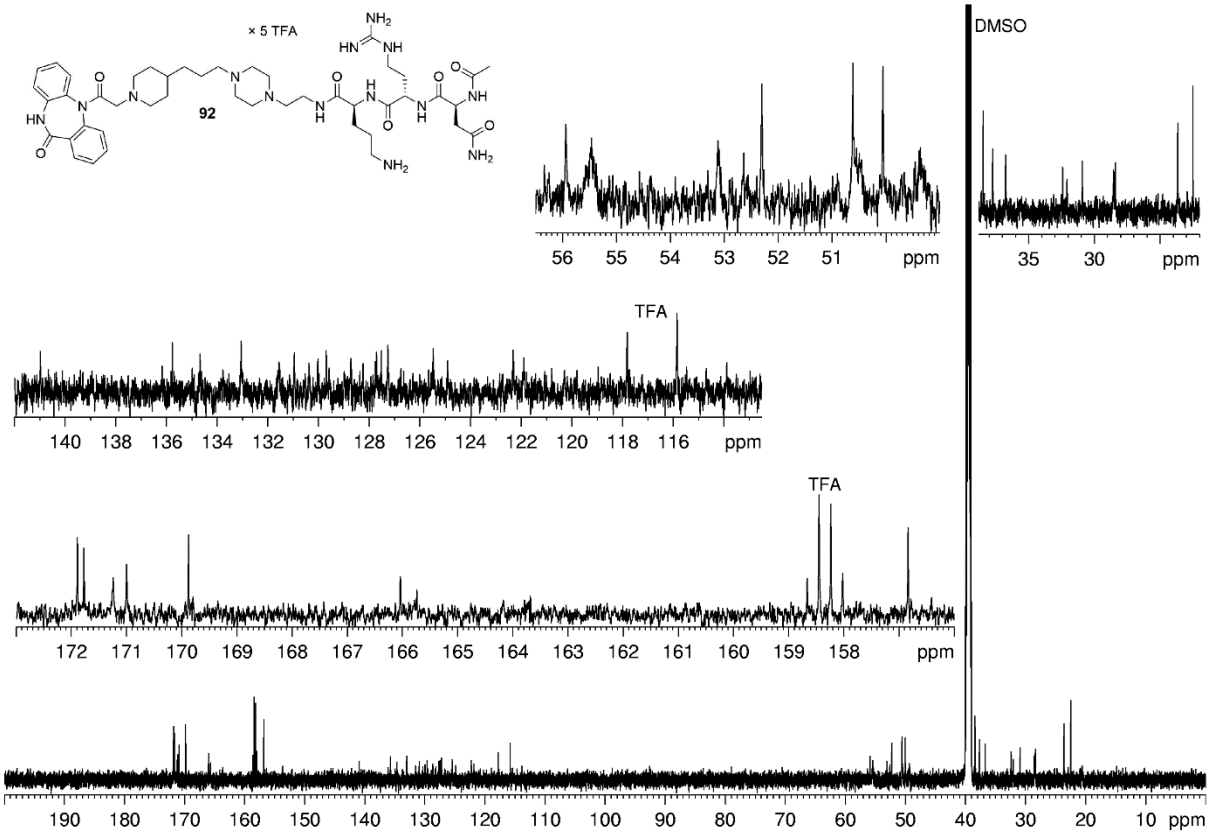
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **91**



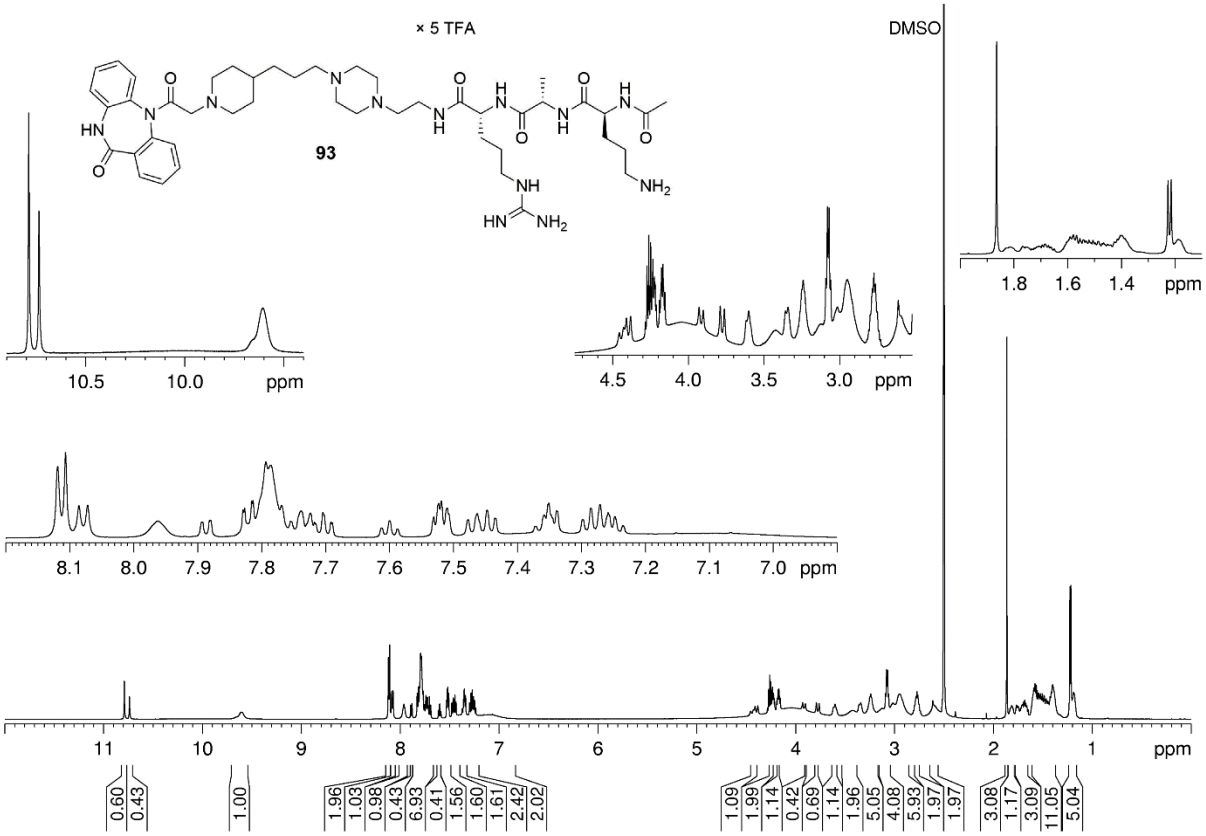
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **92**



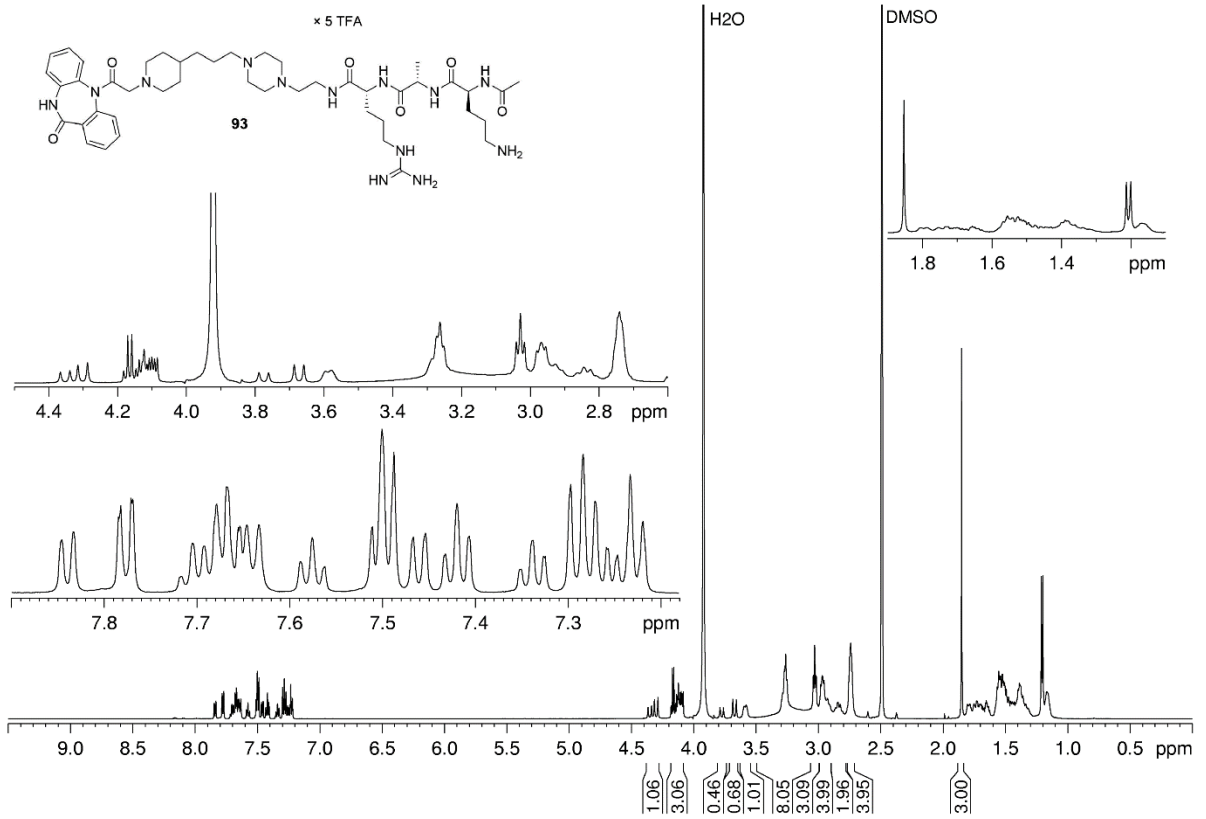
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **92**



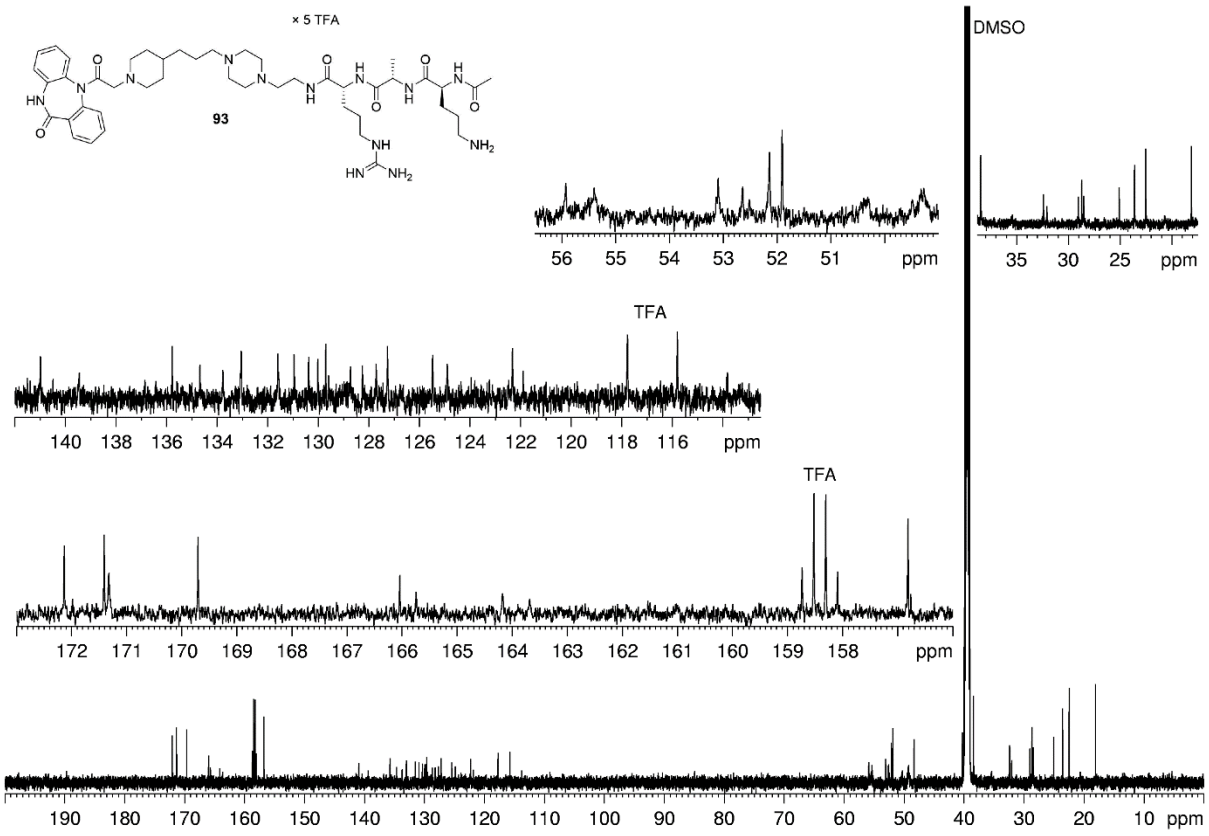
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **92**



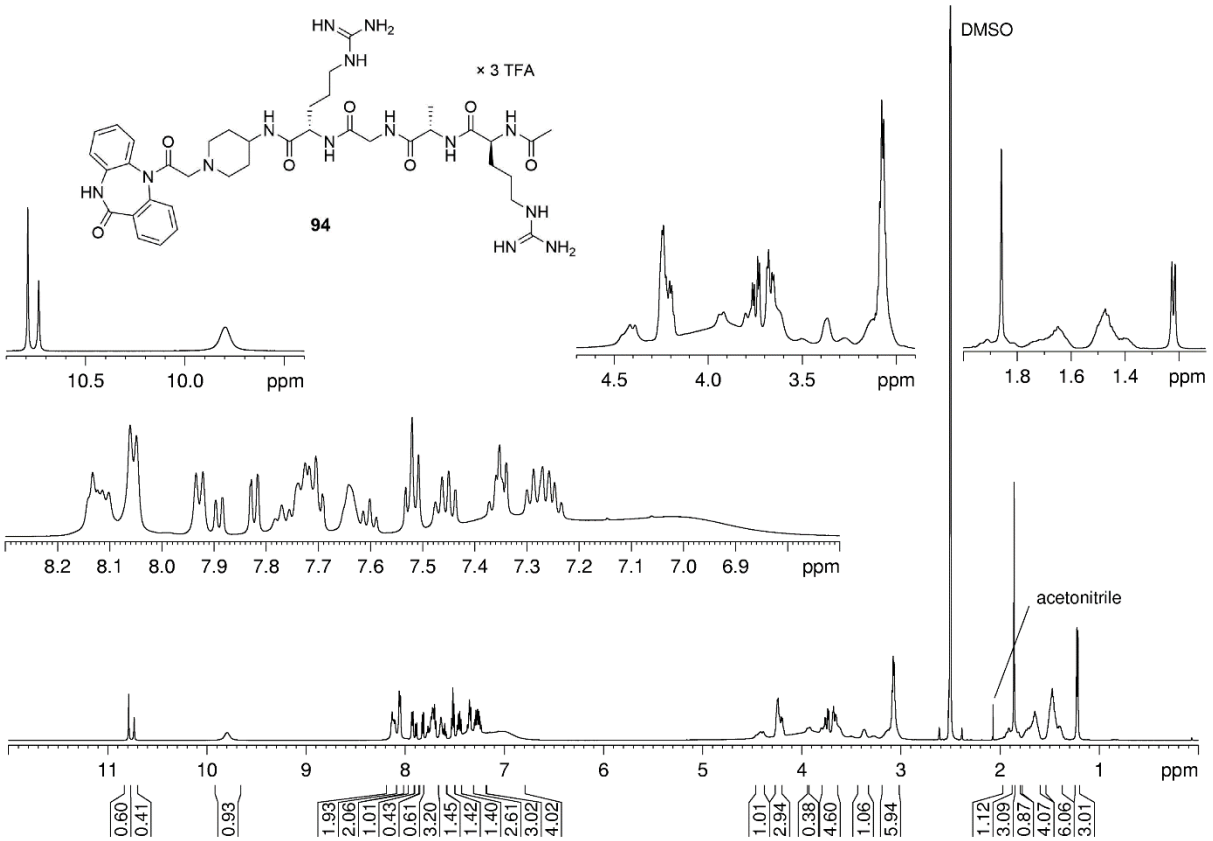
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **93**



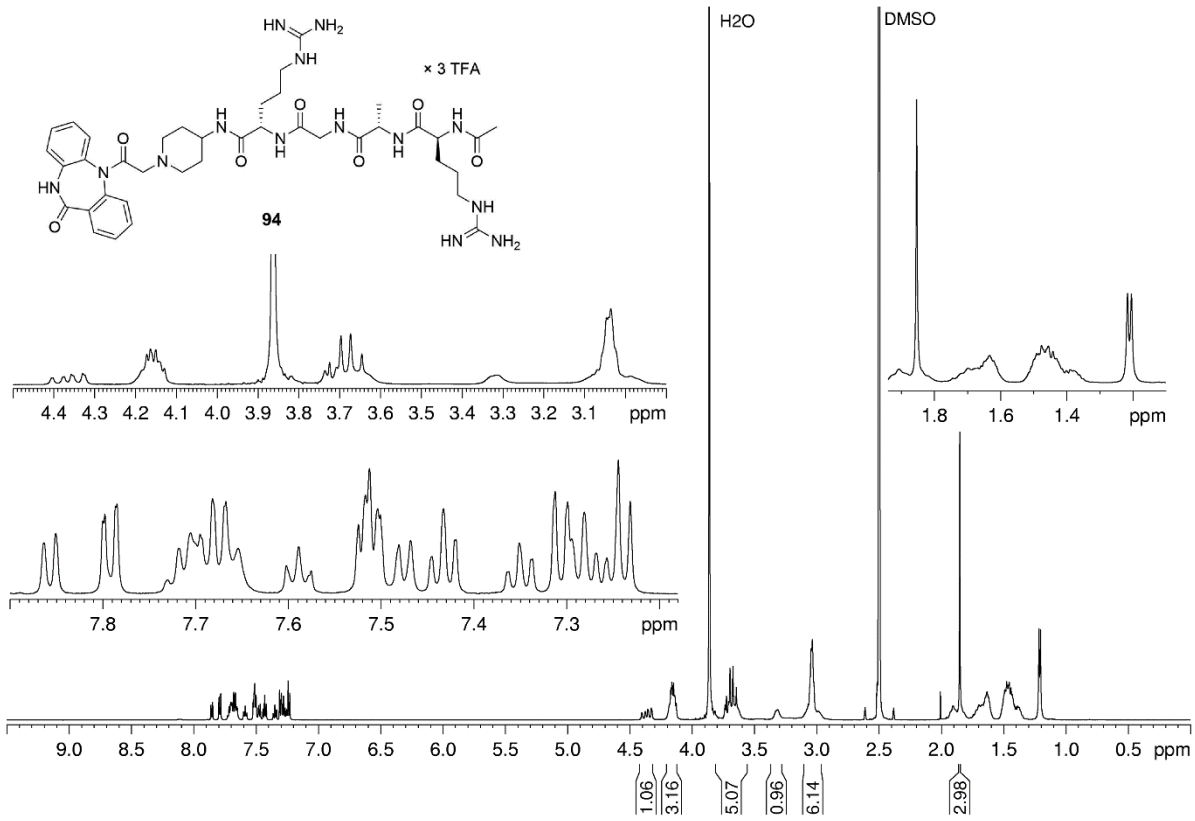
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **93**



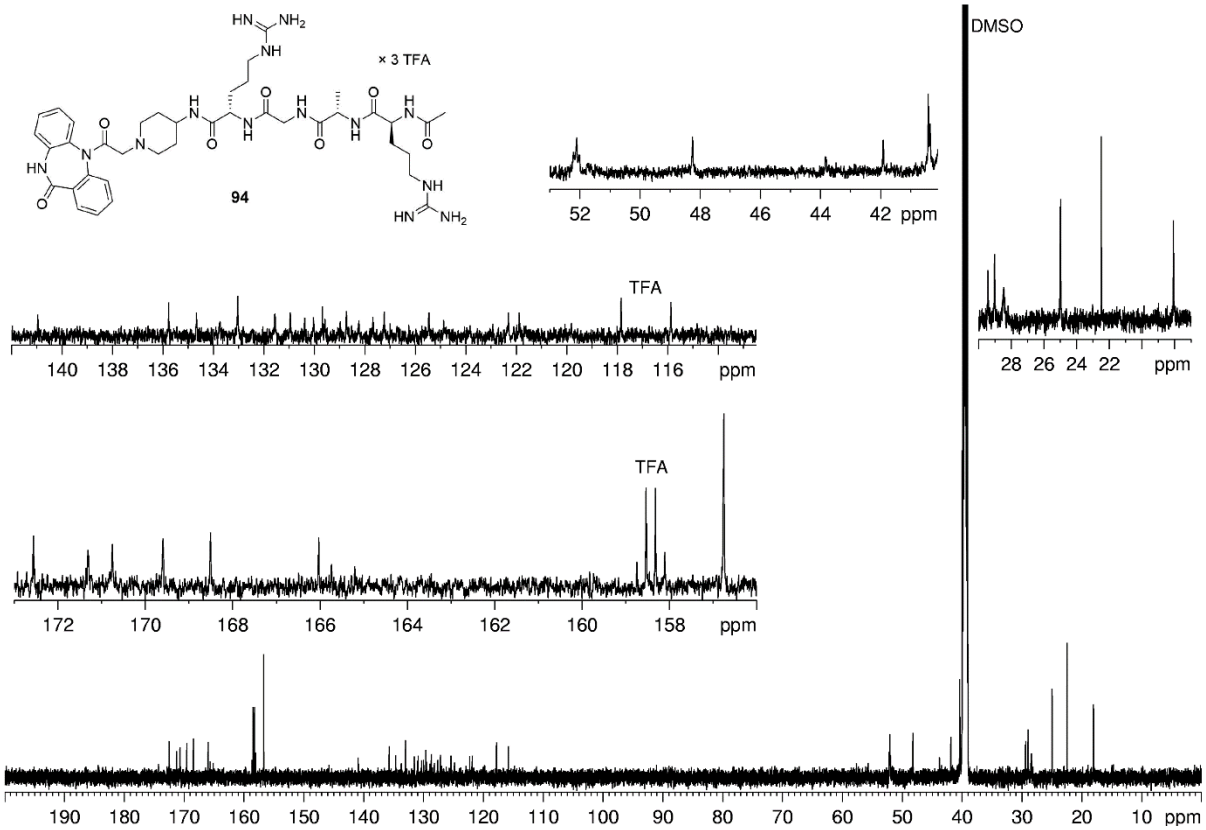
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **93**



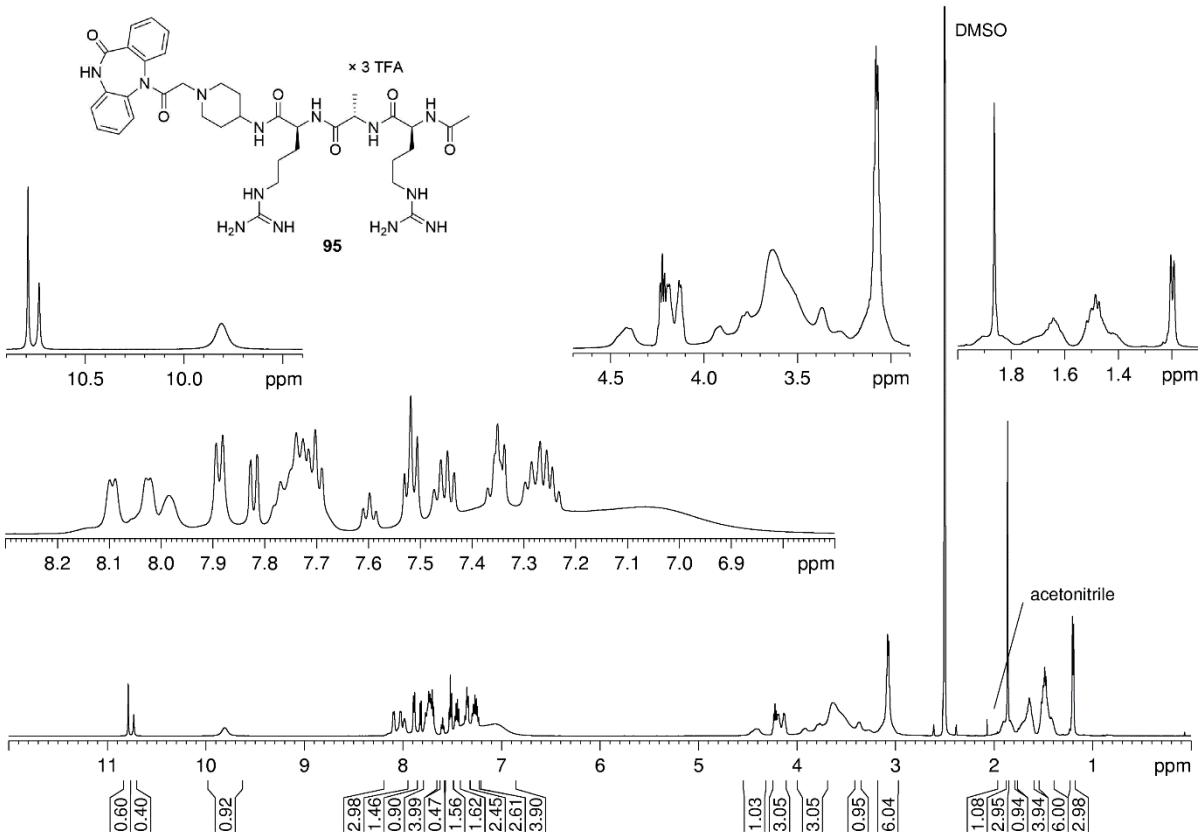
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **94**



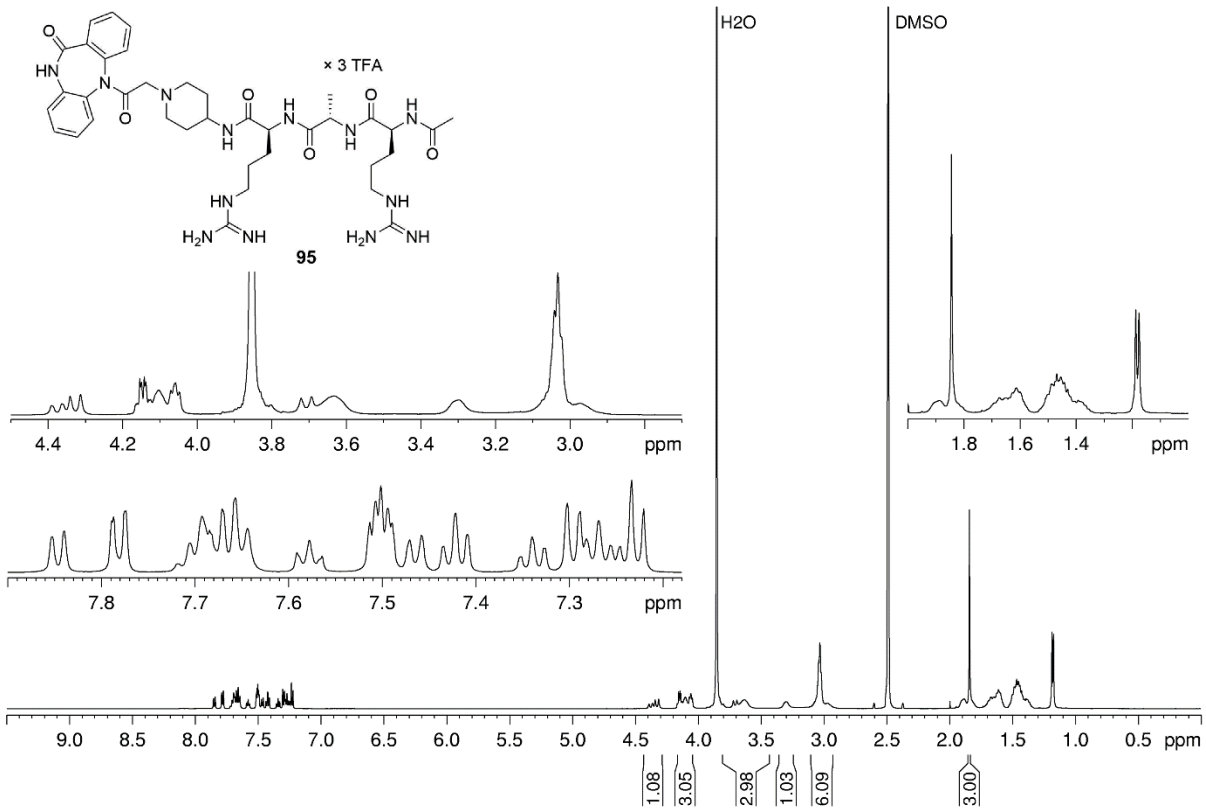
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **94**



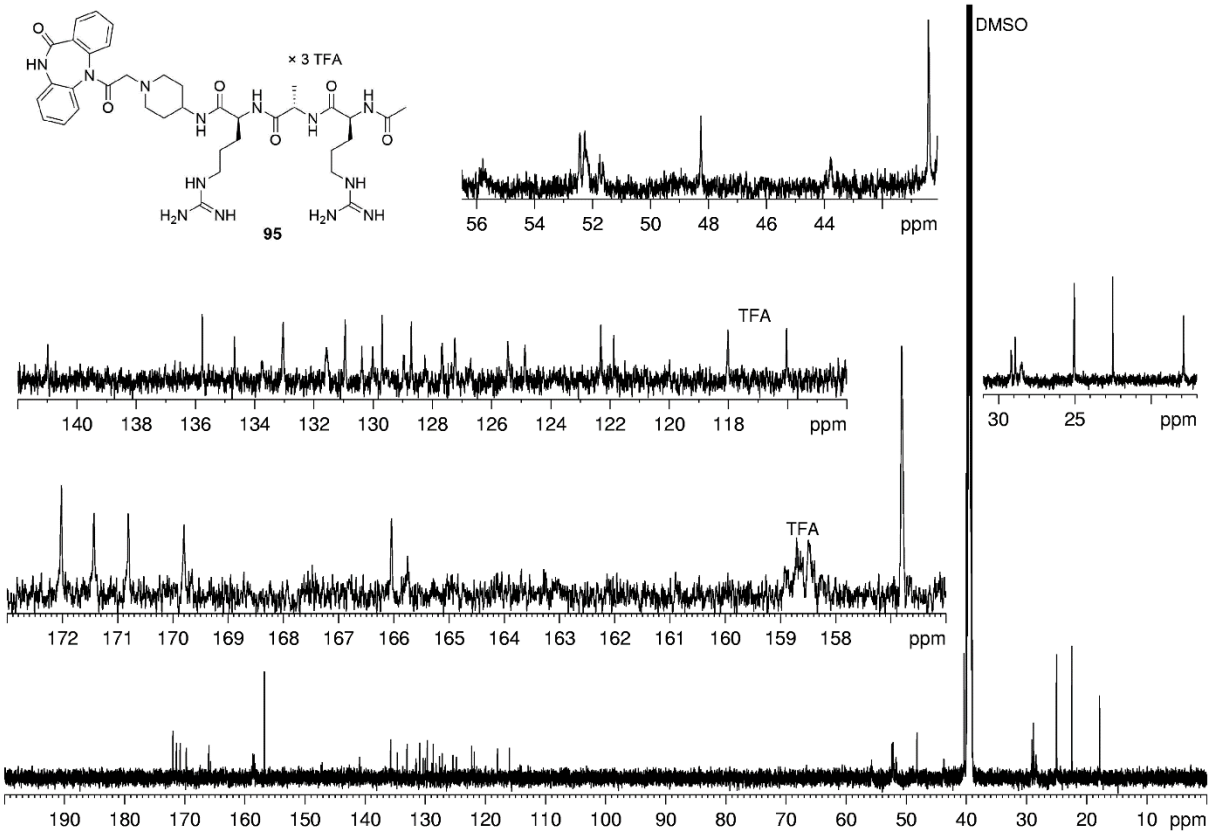
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **94**



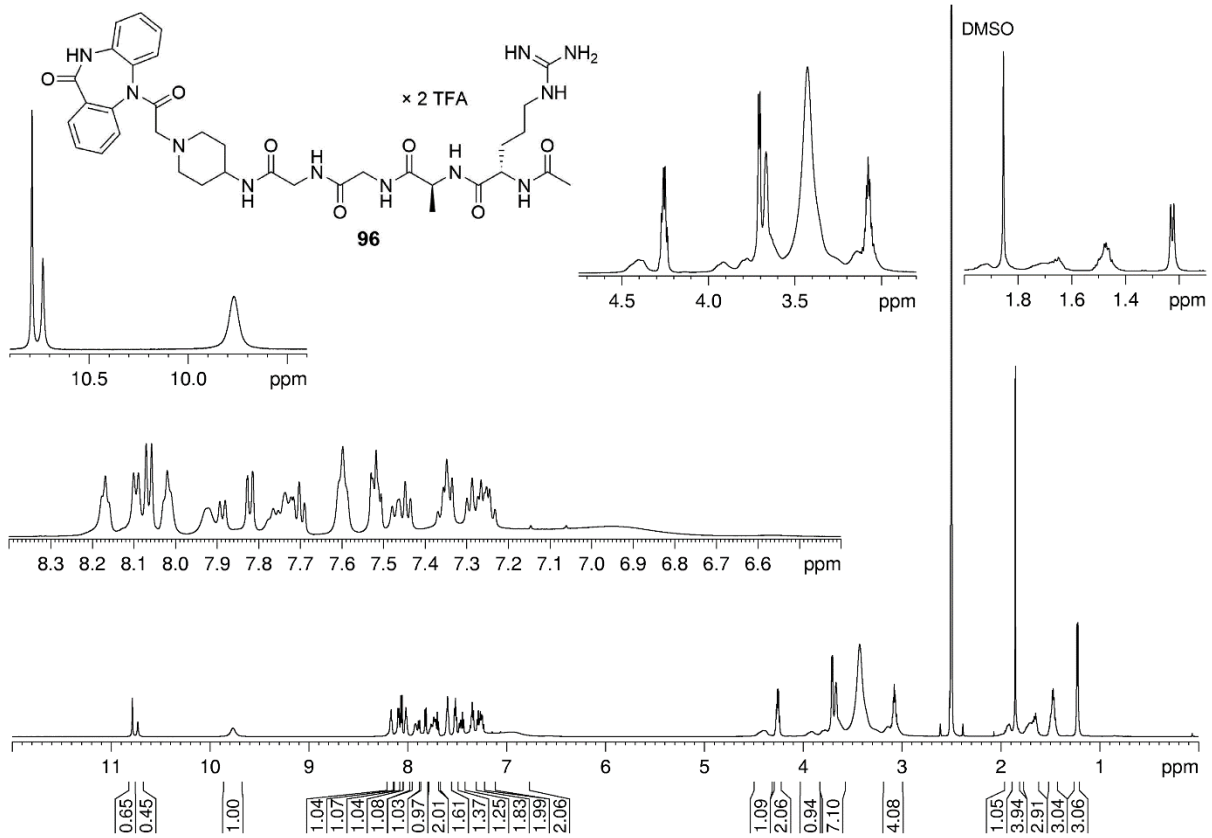
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **95**



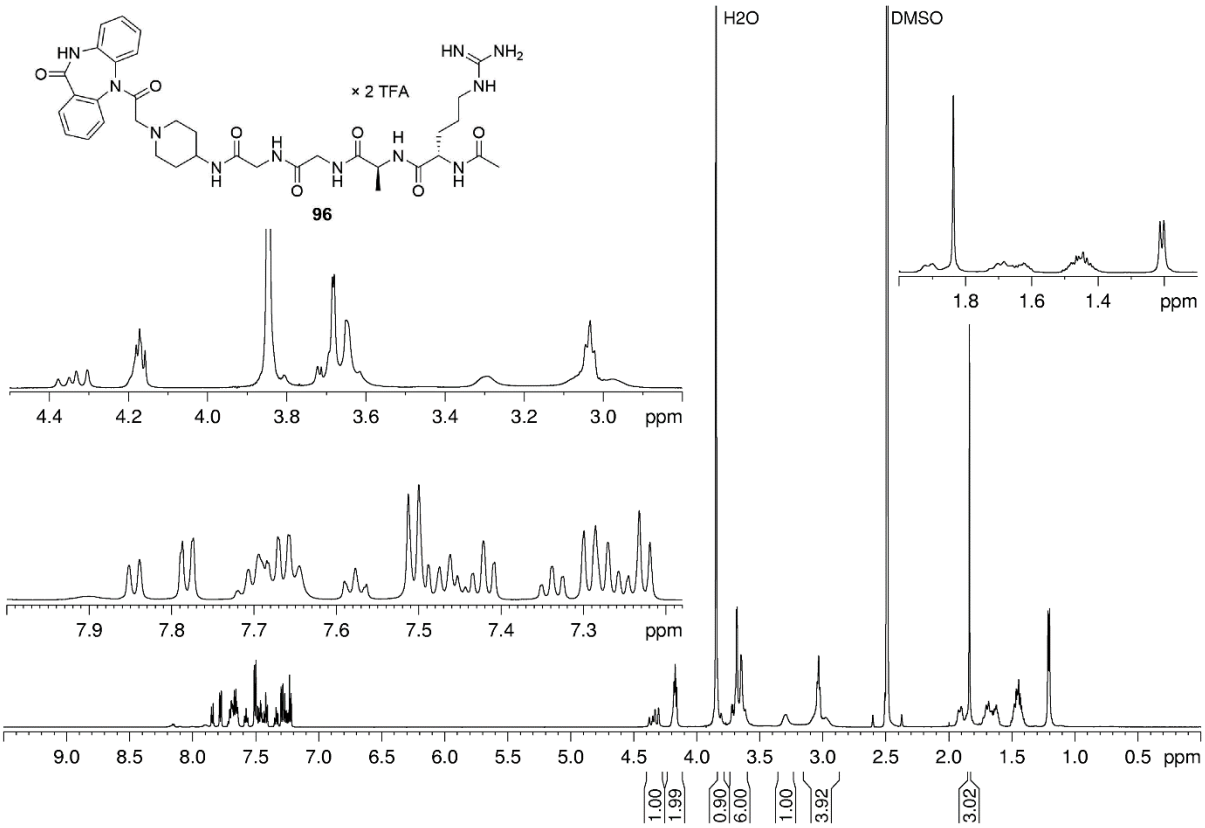
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **95**



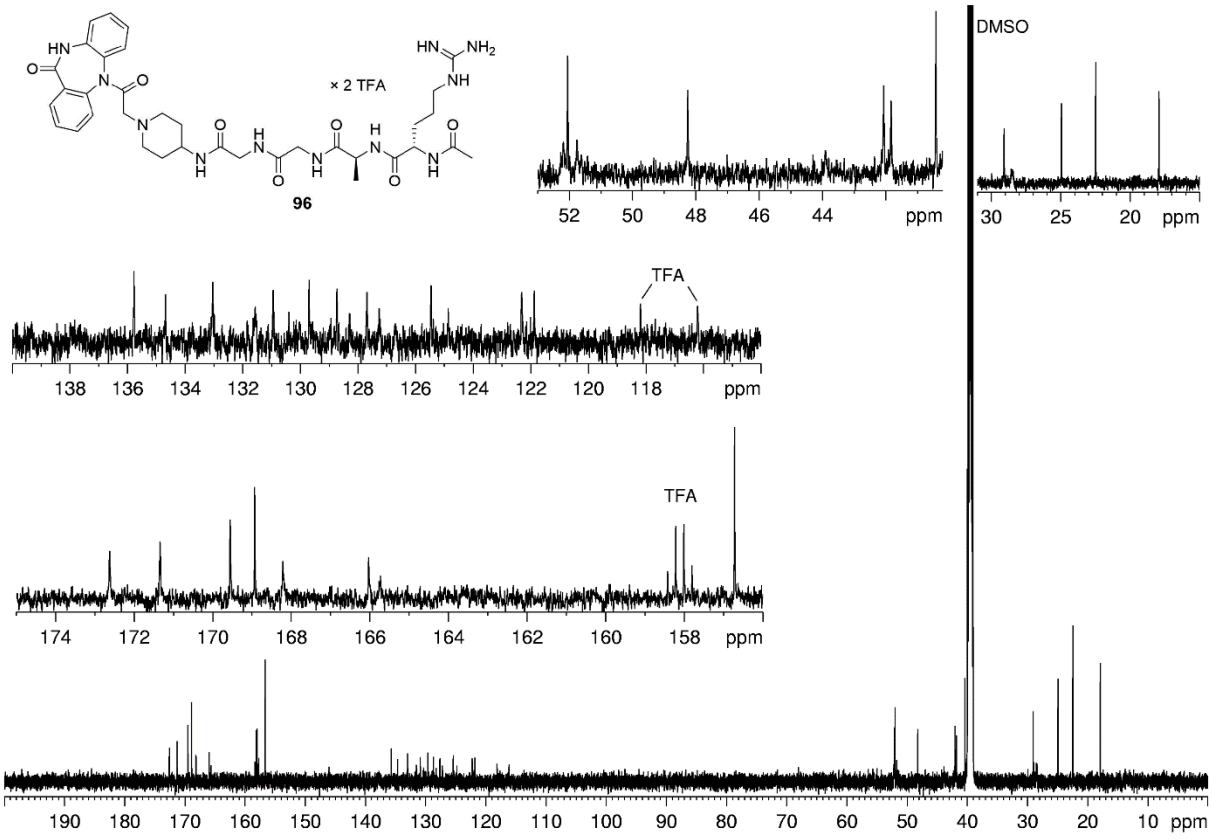
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **95**



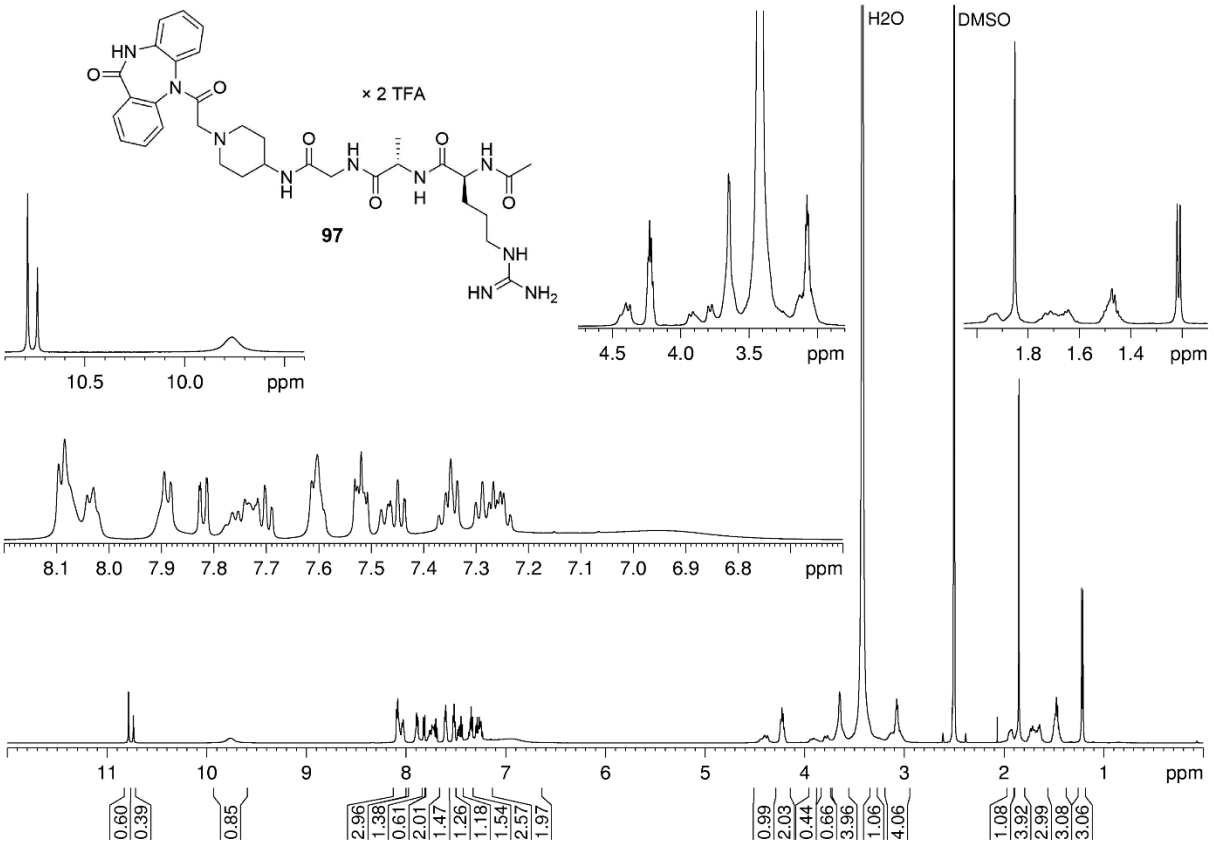
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **96**



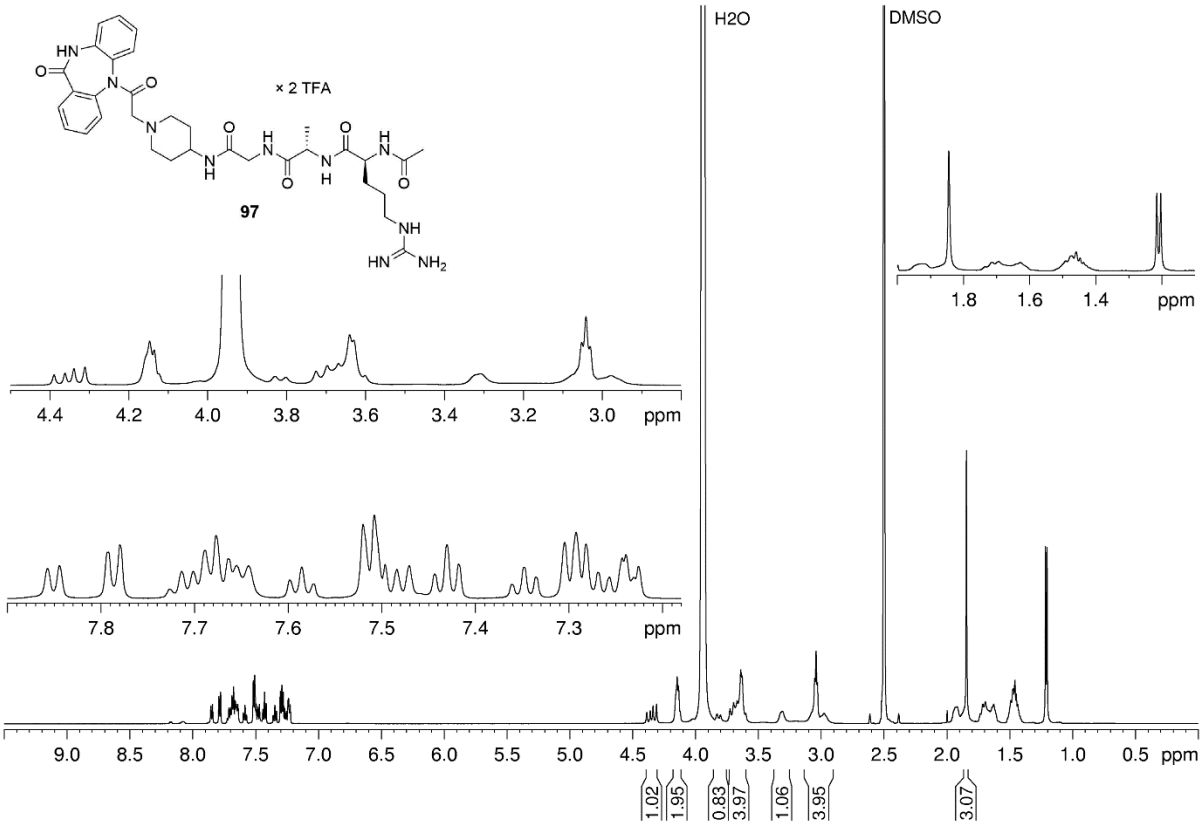
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **96**



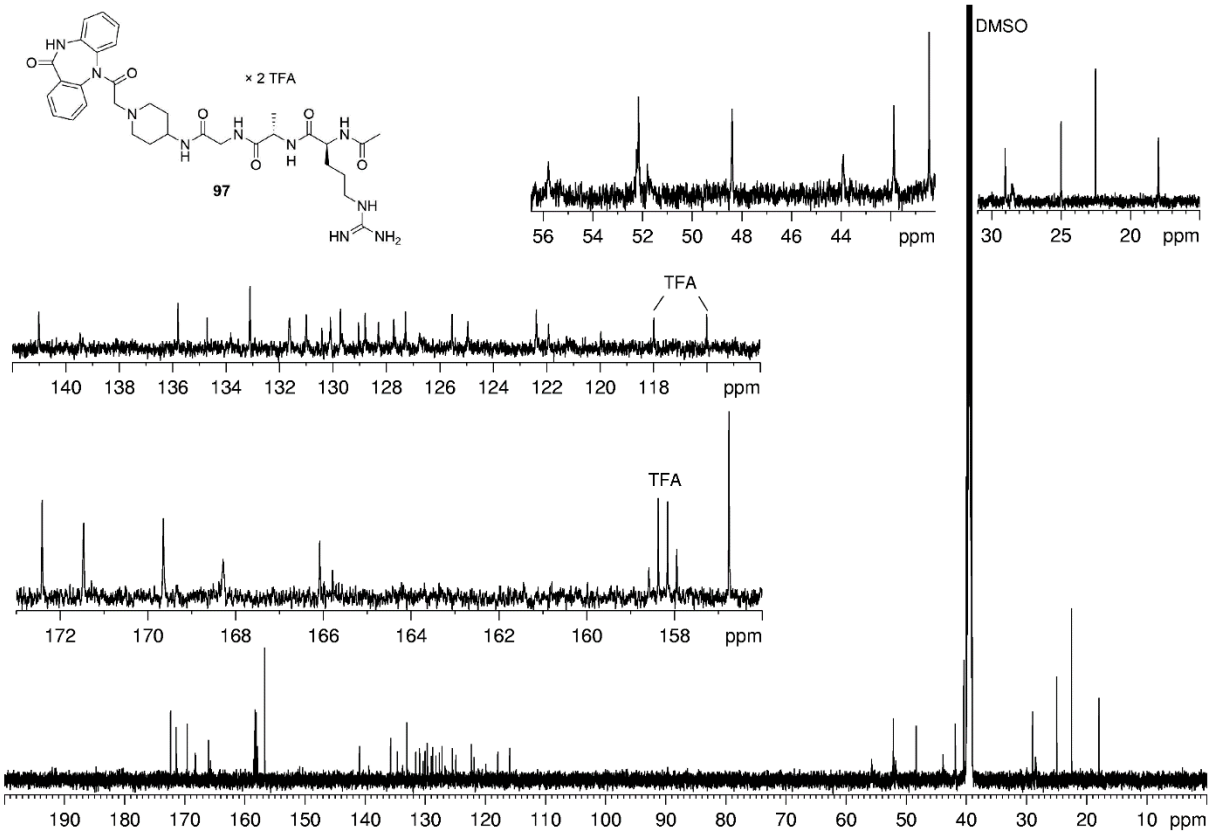
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **96**



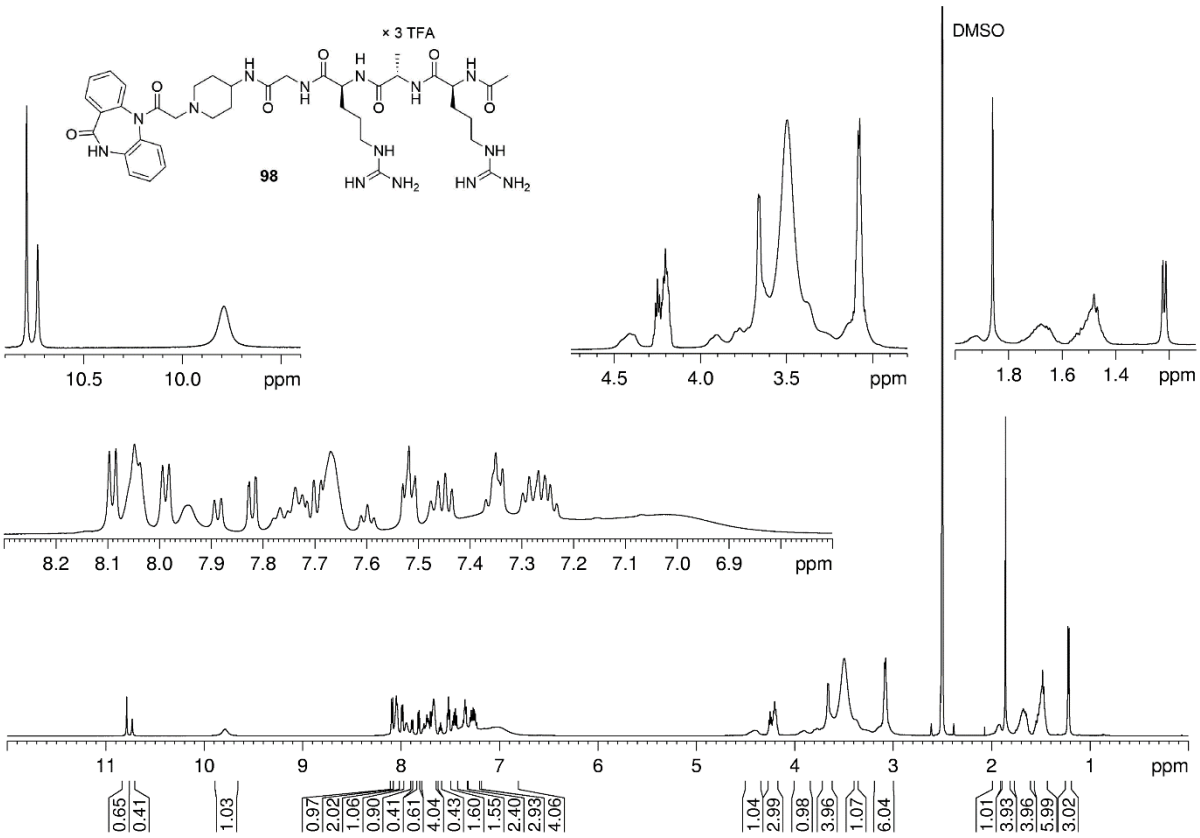
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **97**



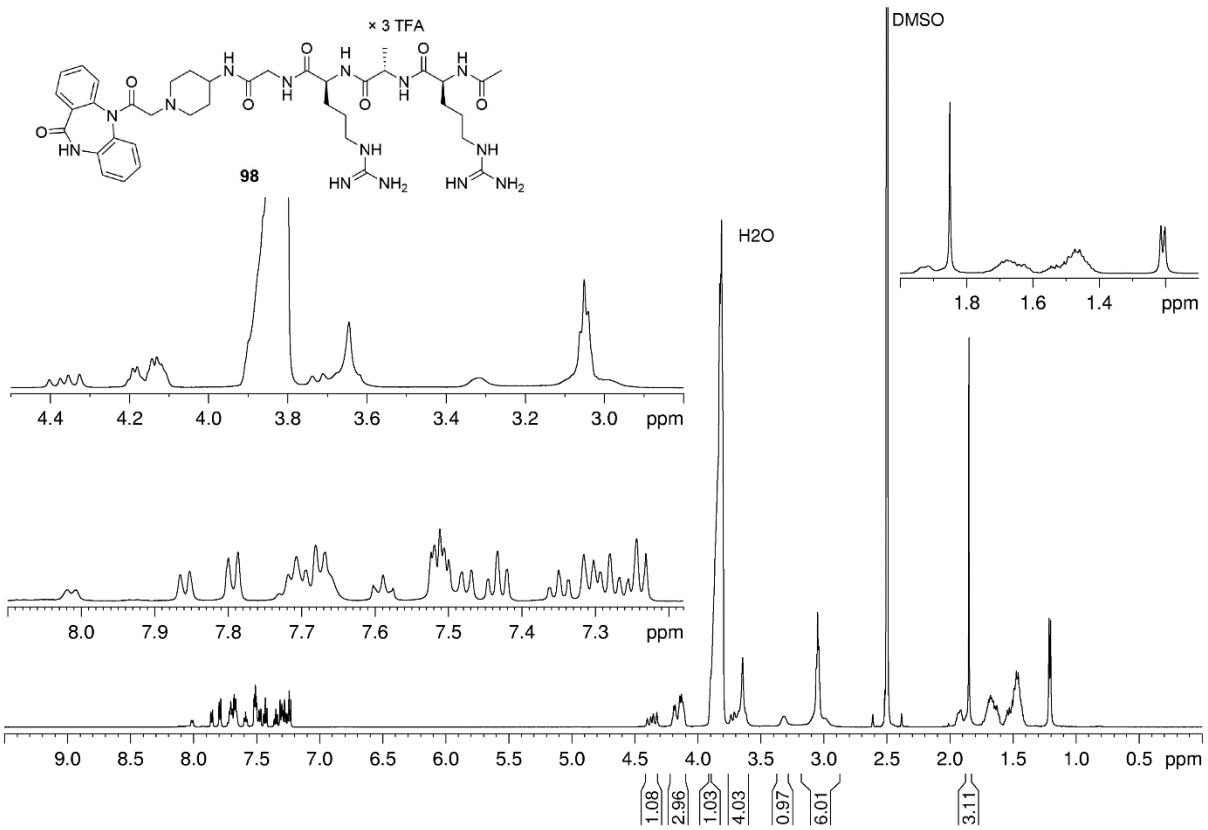
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **97**



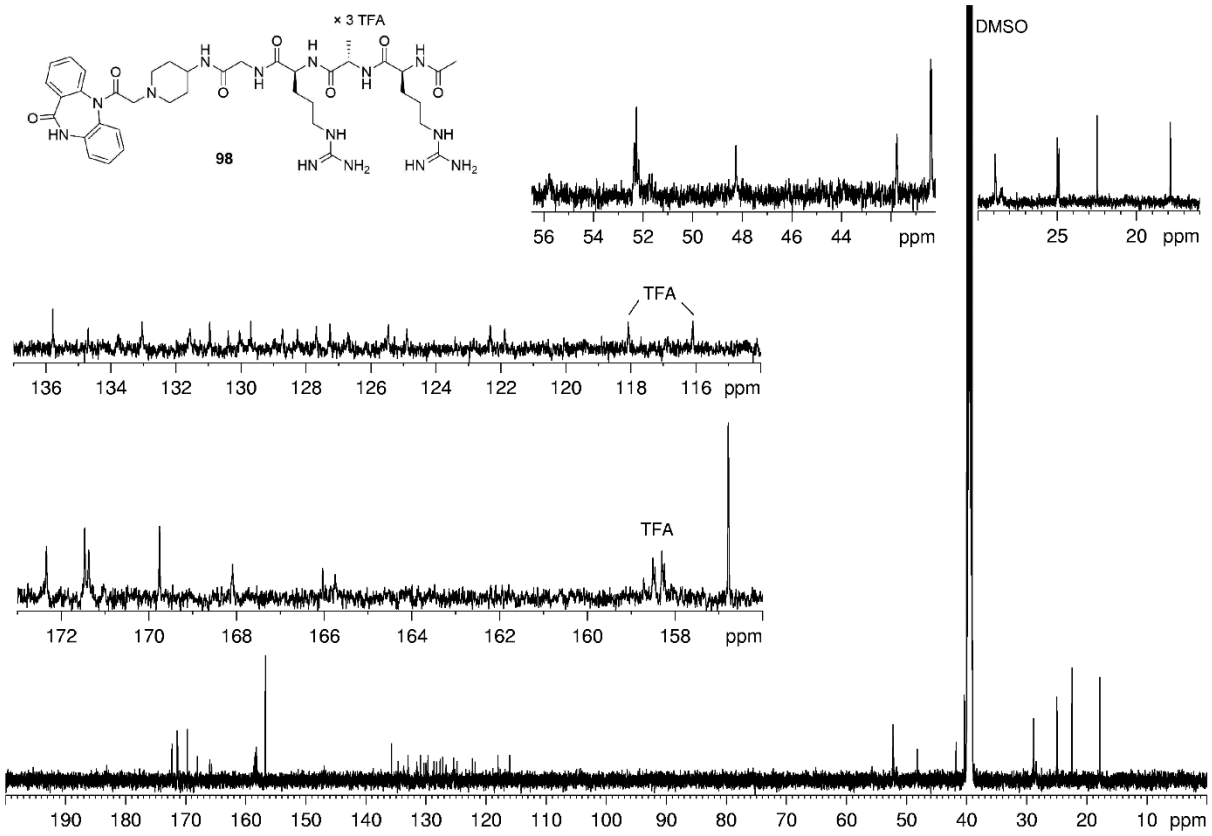
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **97**



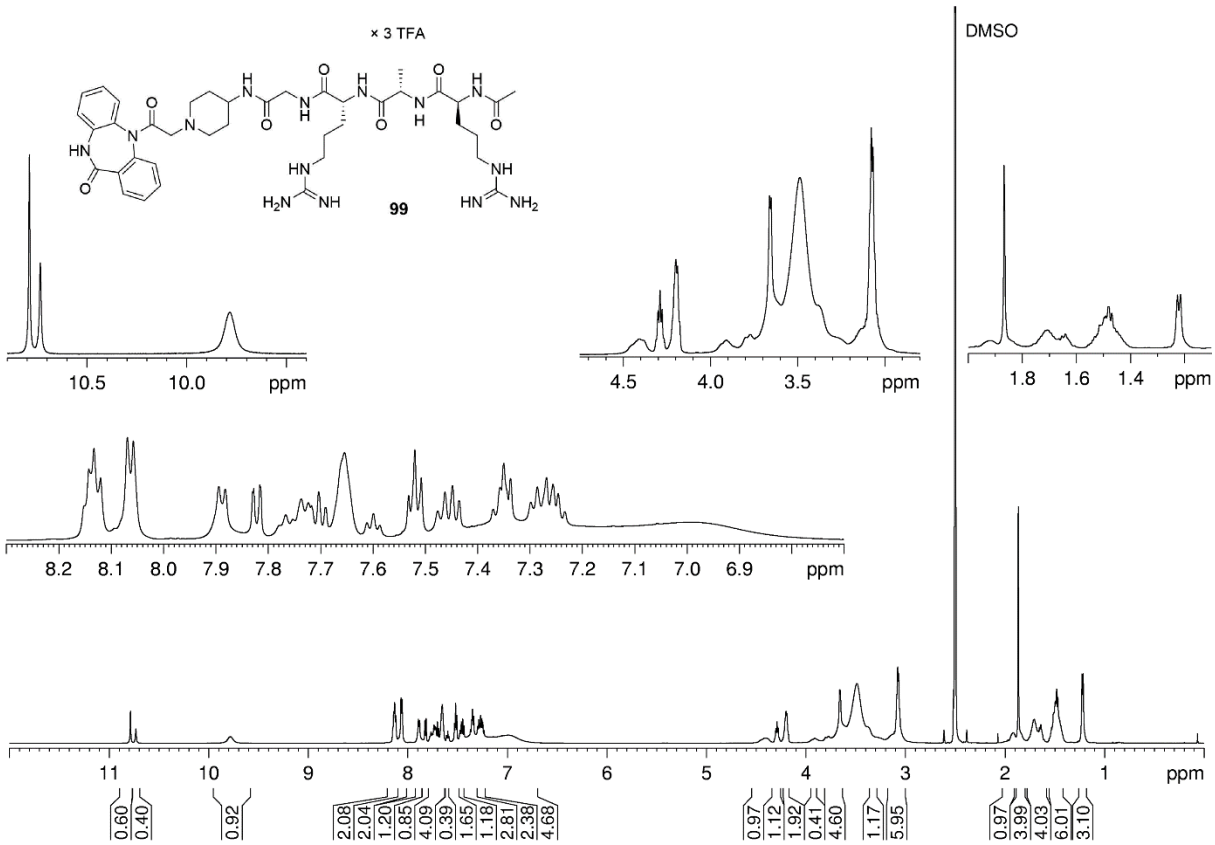
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **98**



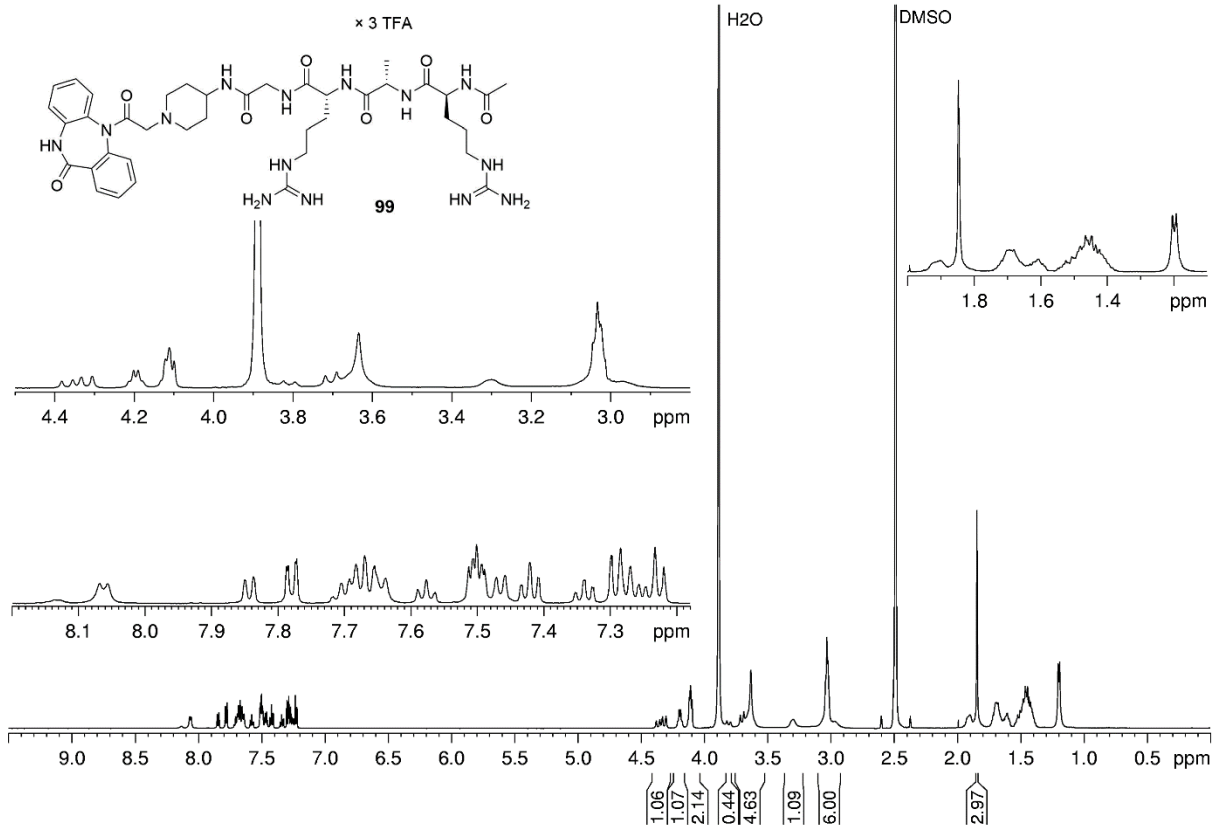
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **98**



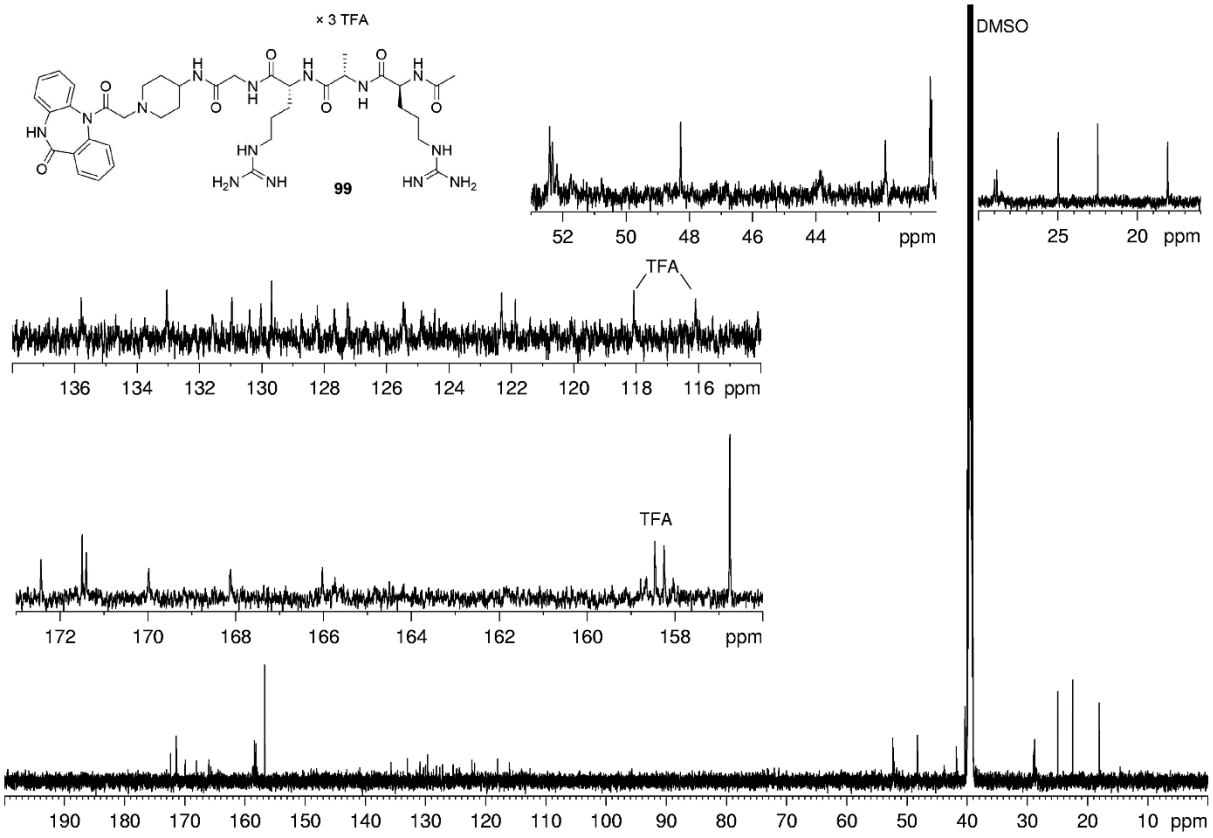
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **98**



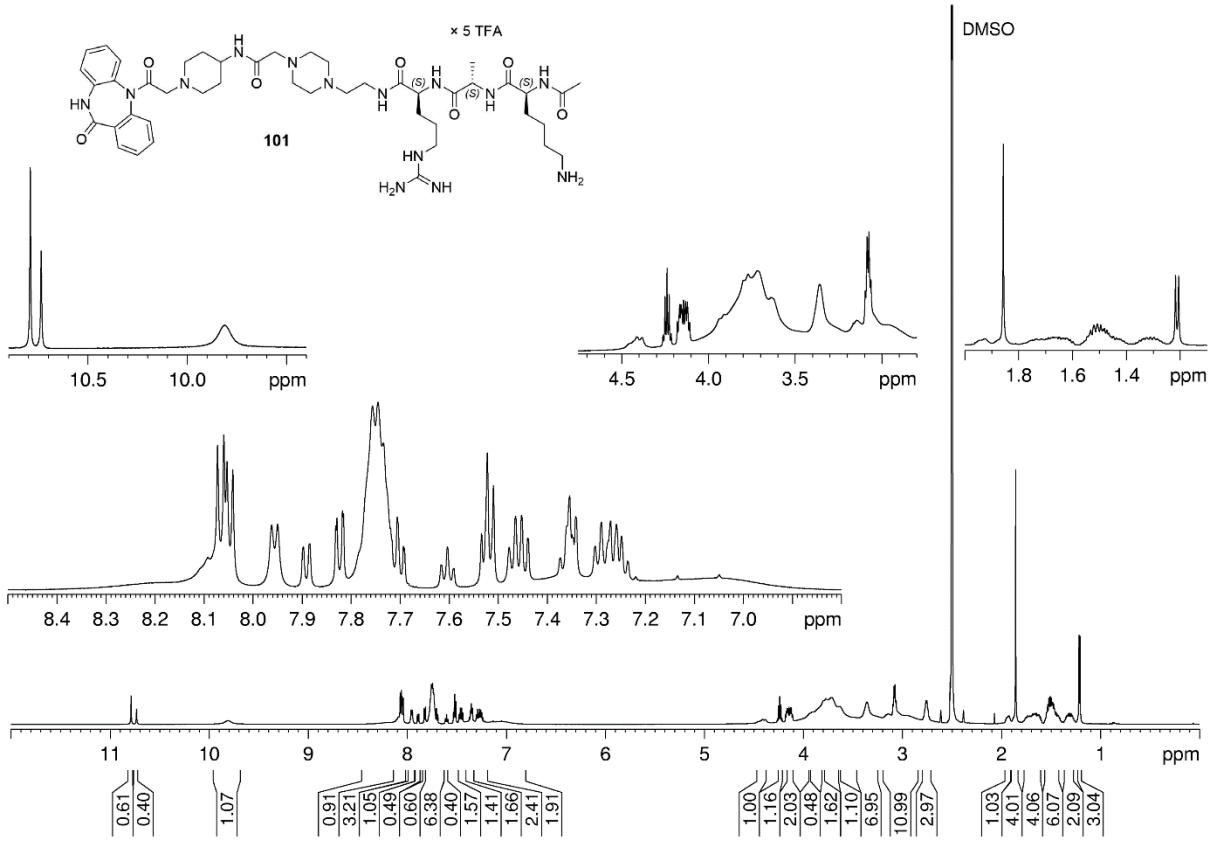
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **99**



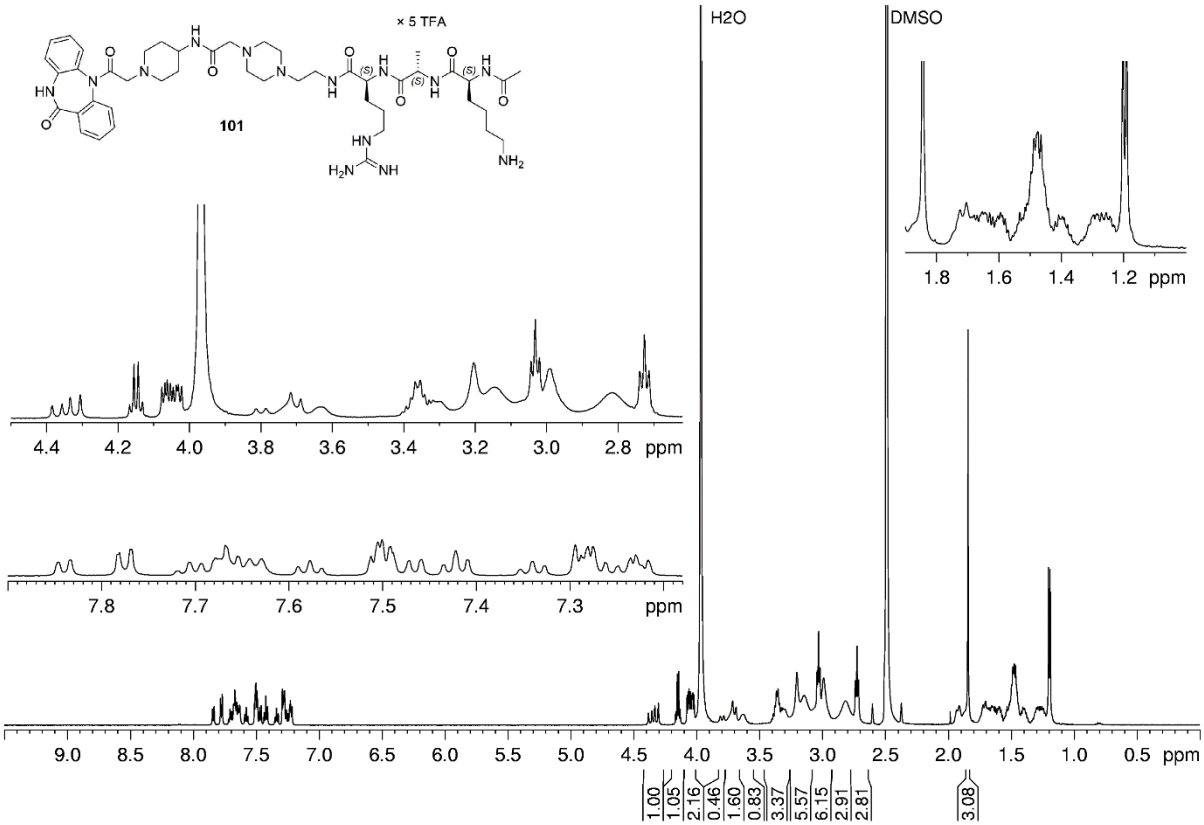
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **99**



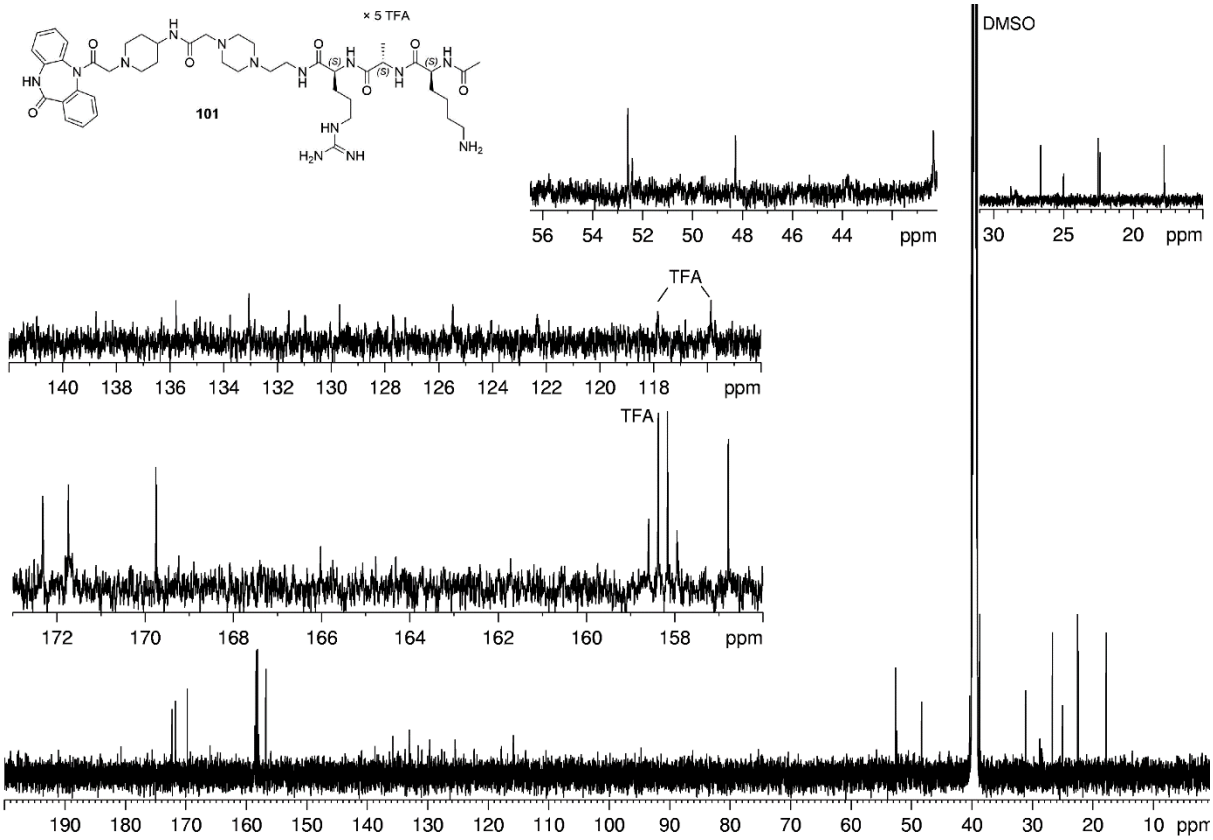
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **99**



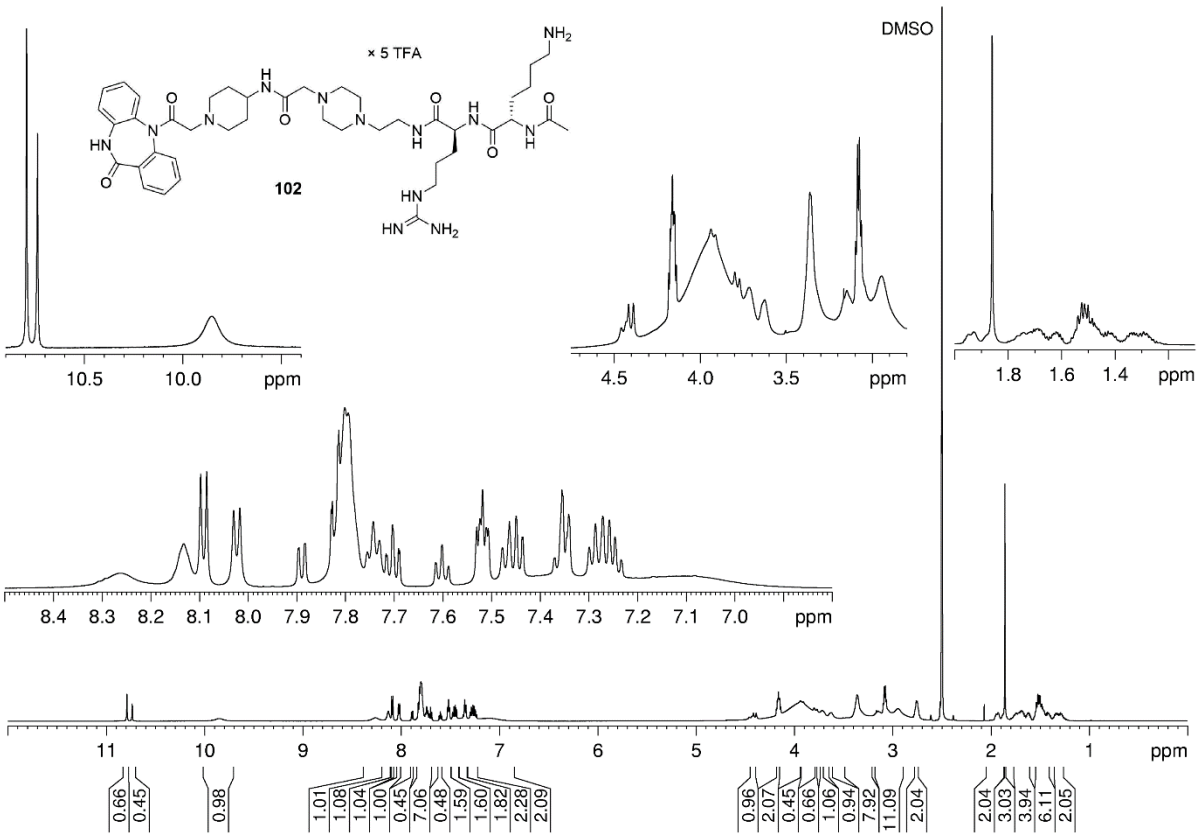
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **101**



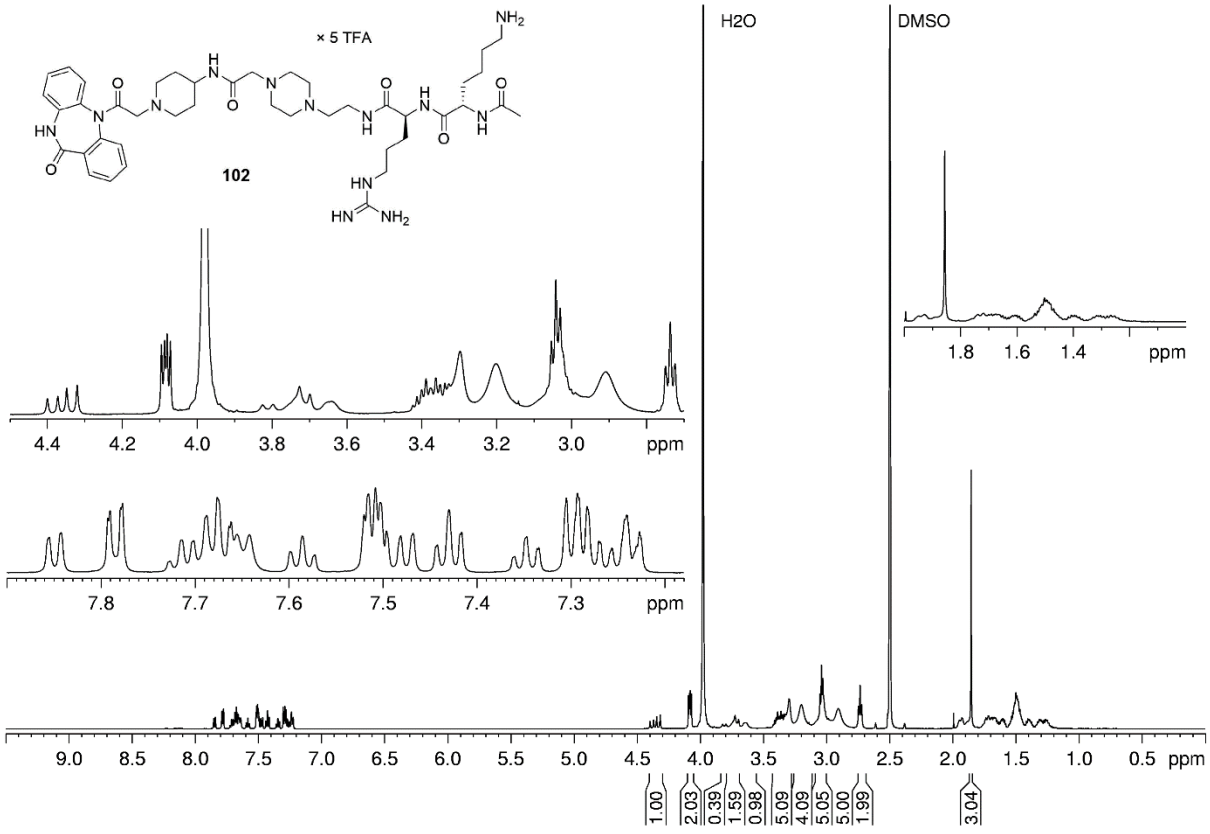
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **101**



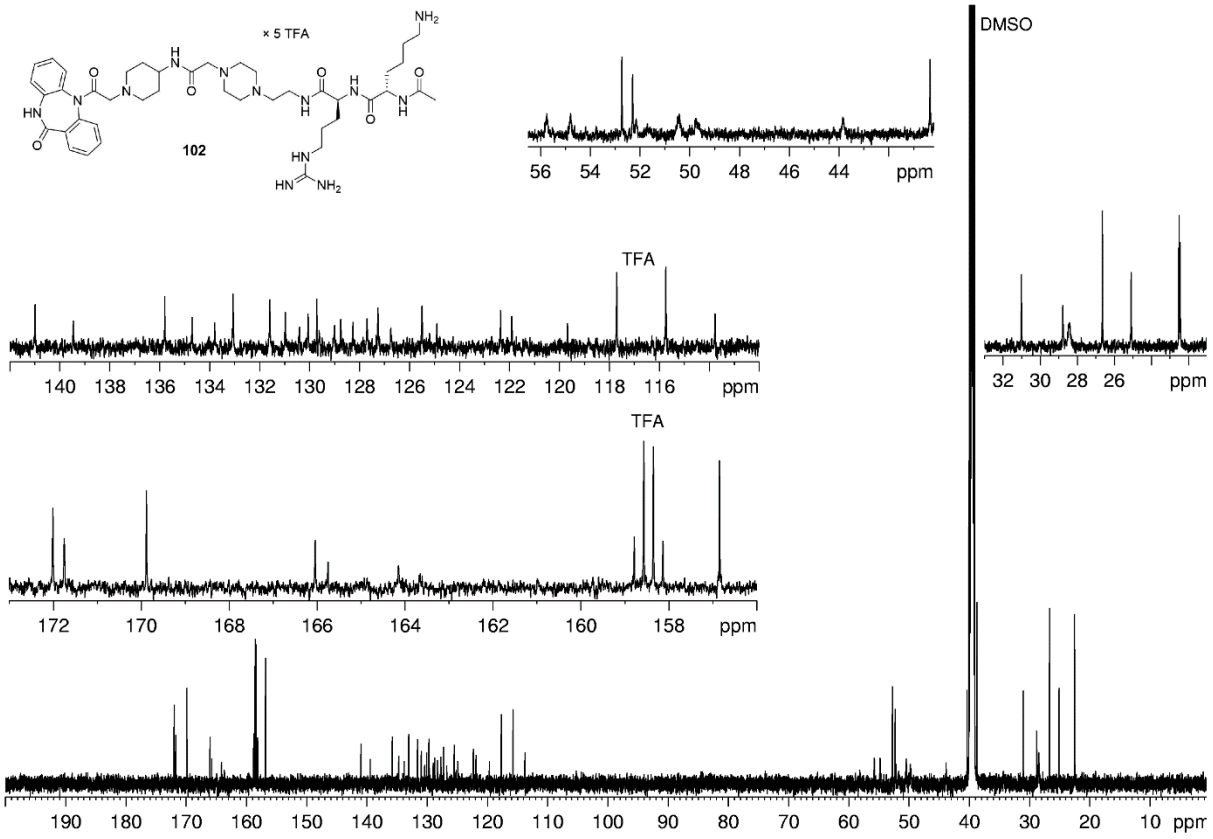
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **101**



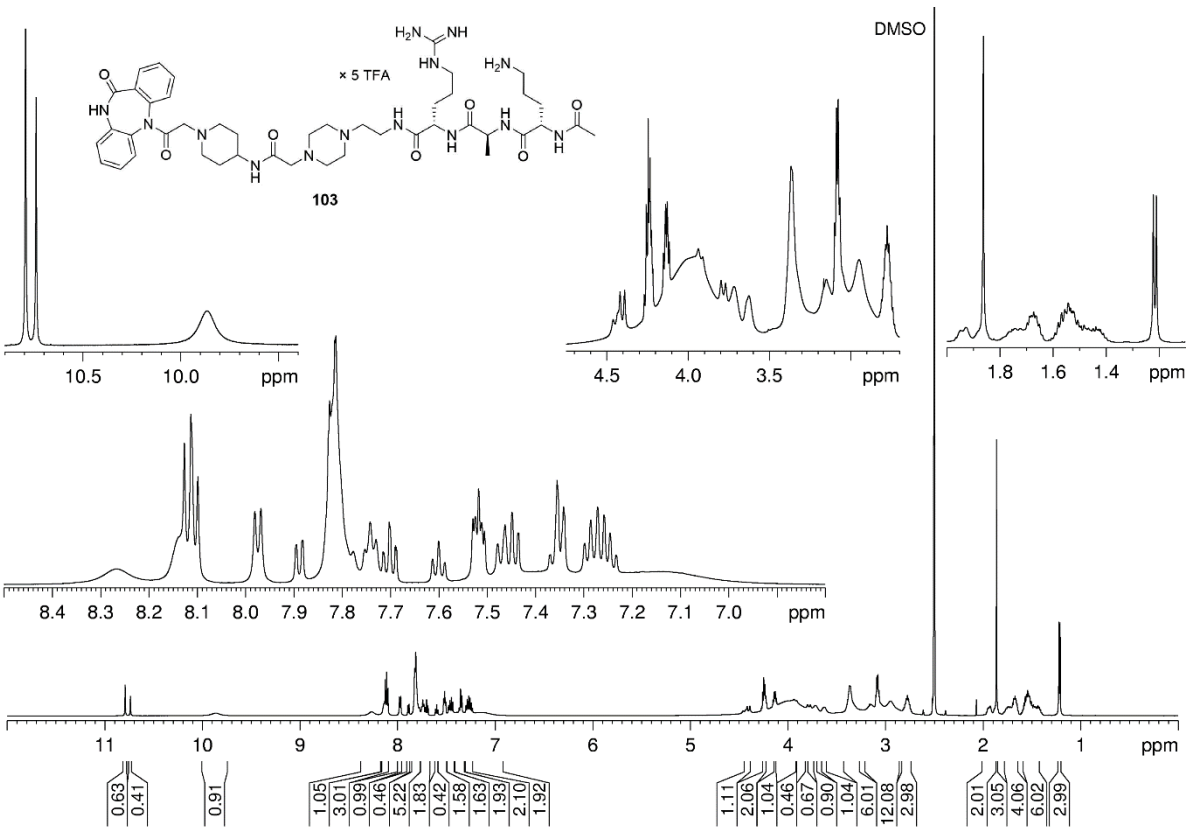
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **102**



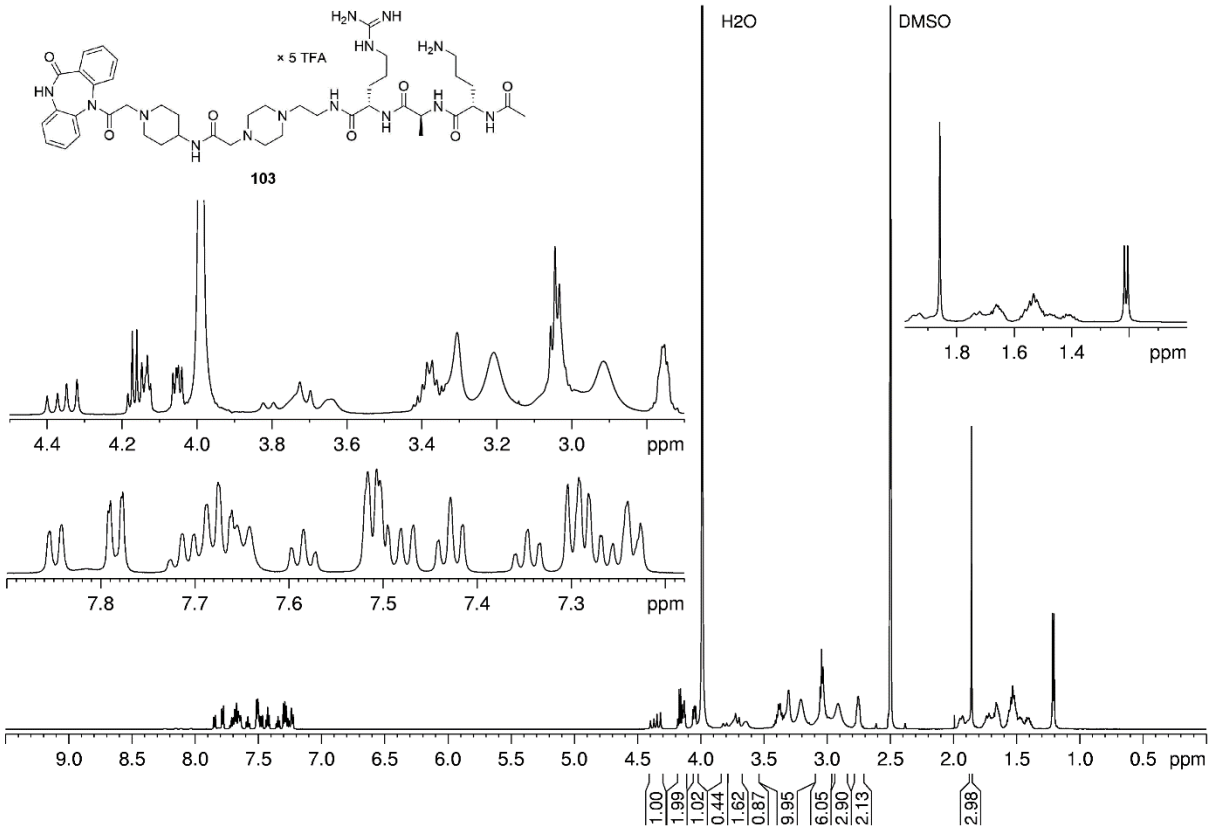
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **102**



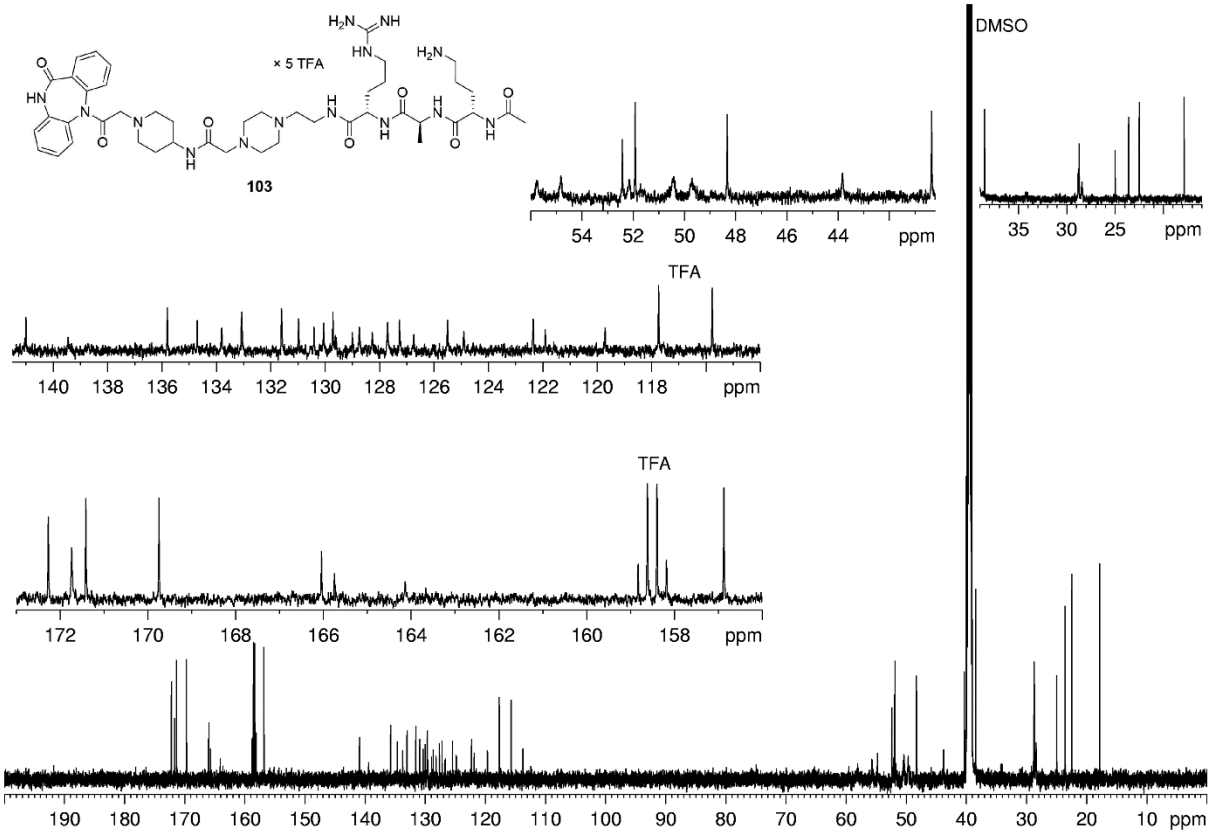
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **102**



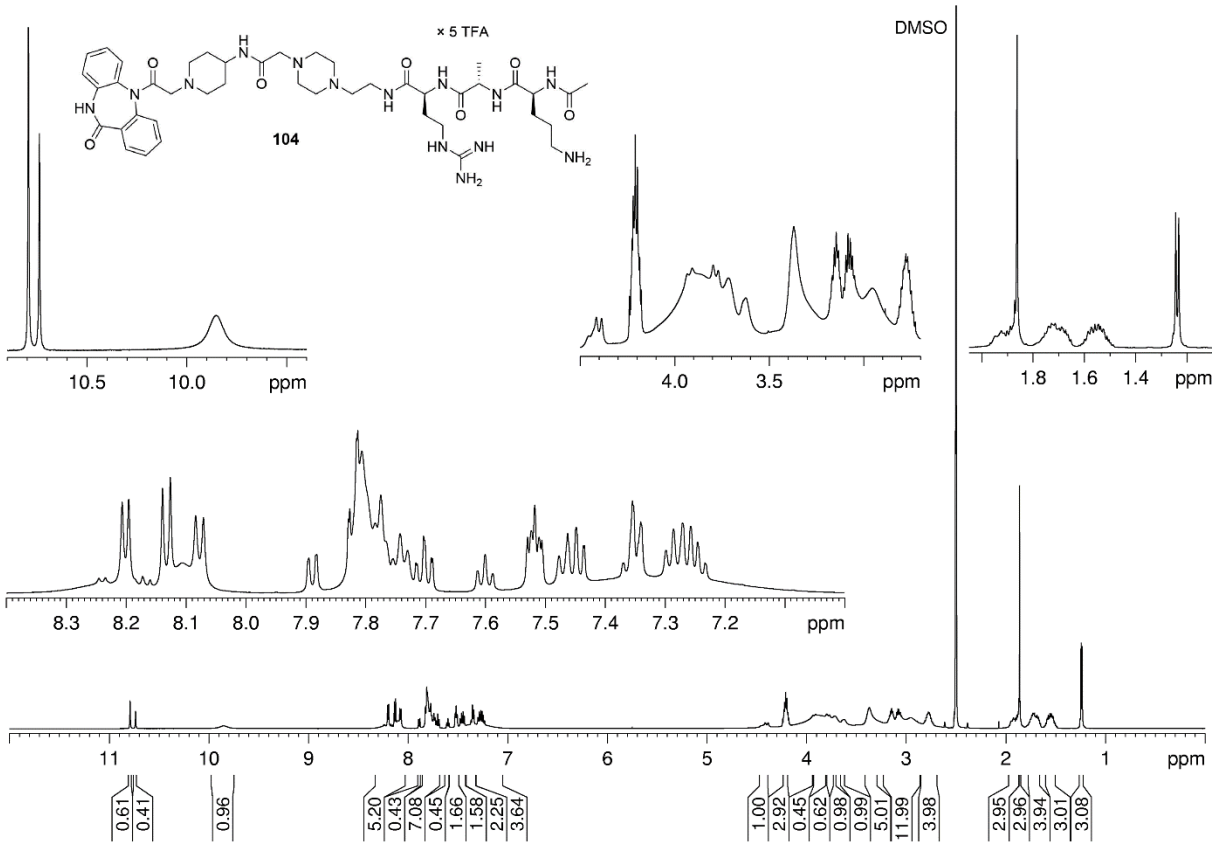
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **103**



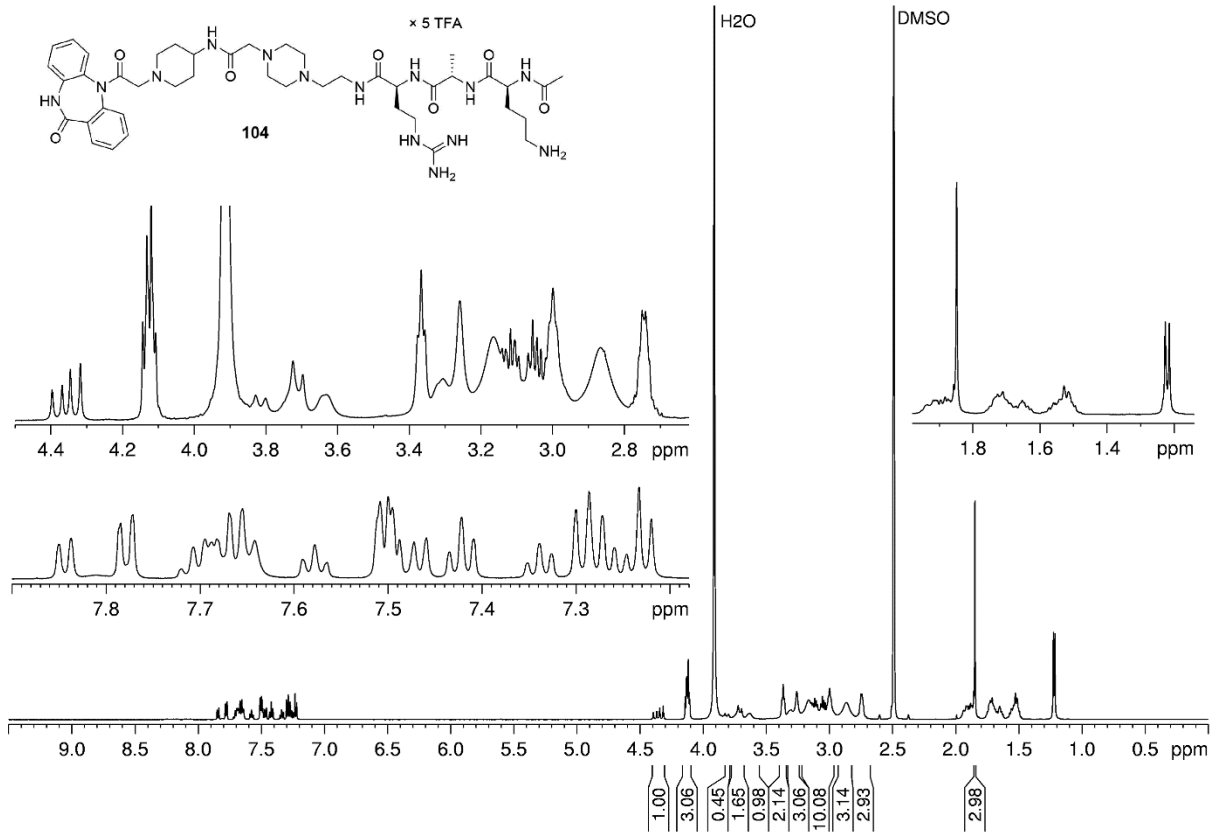
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **103**



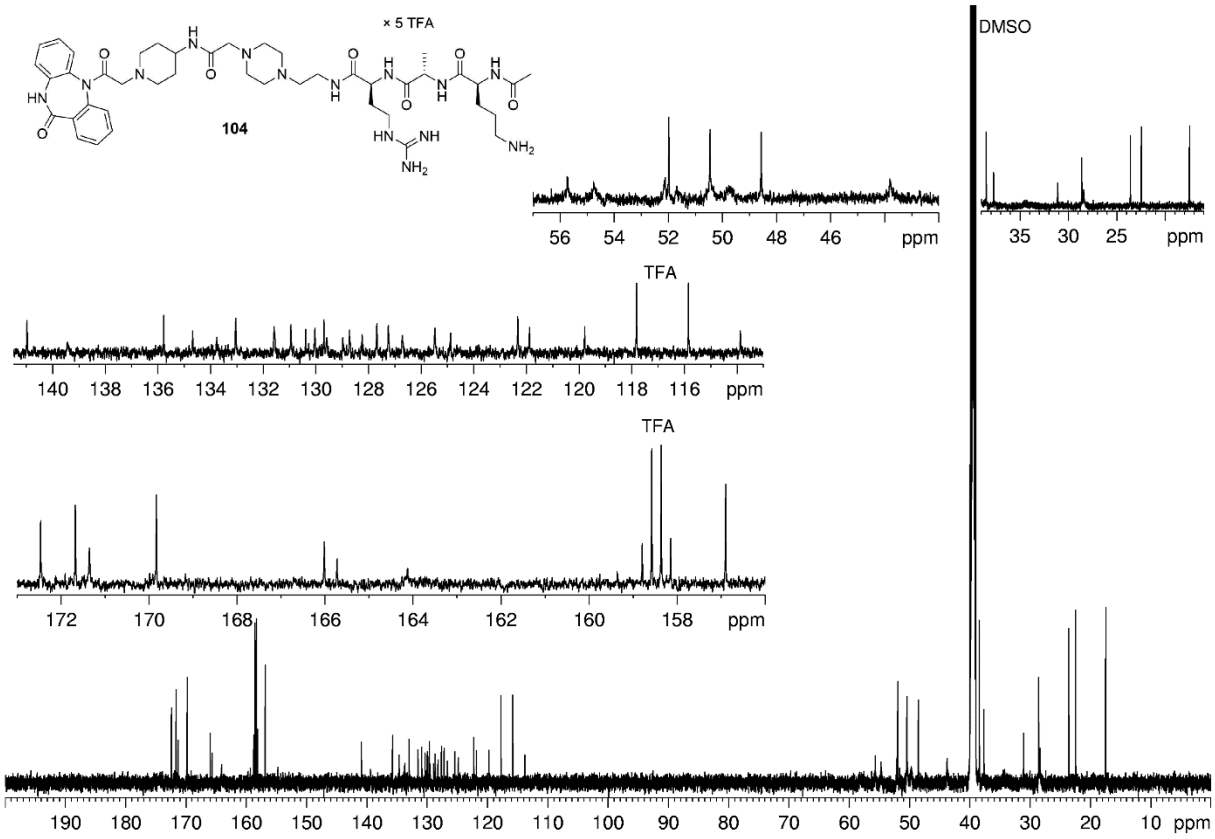
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **103**



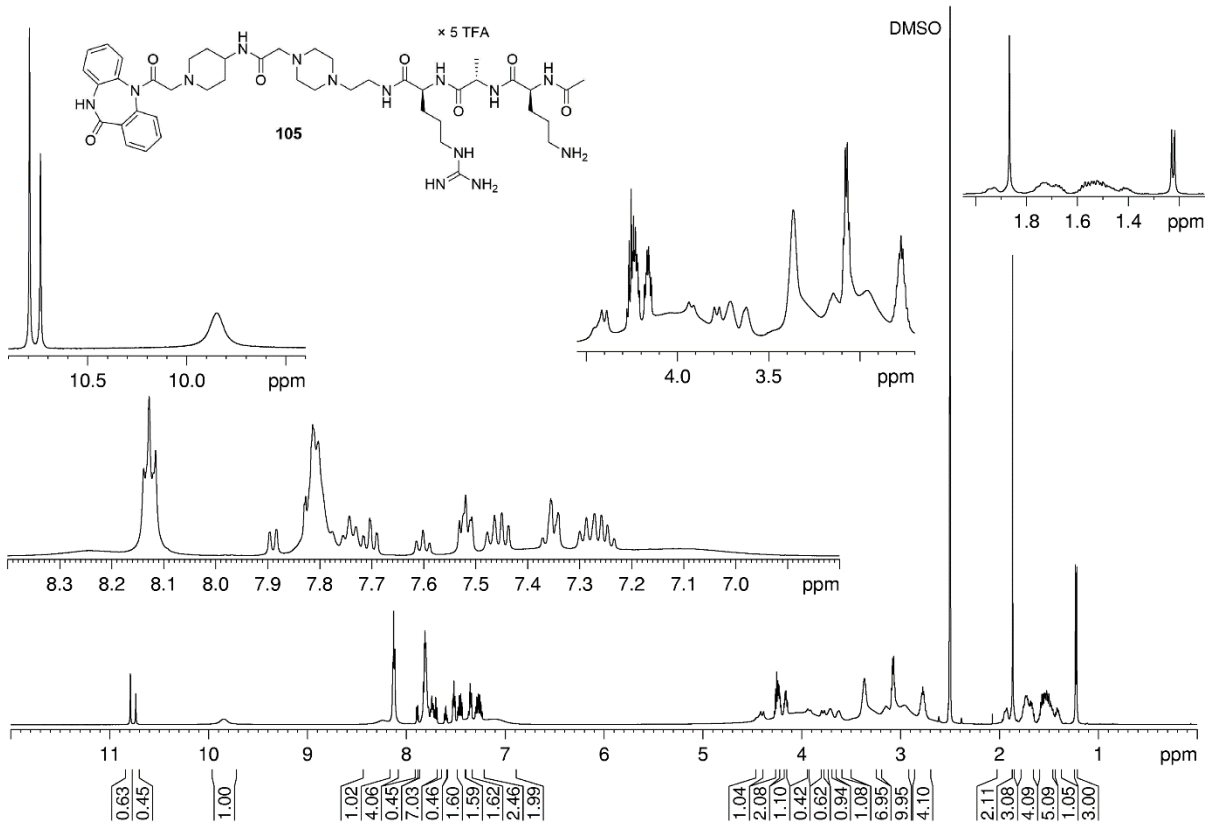
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **104**



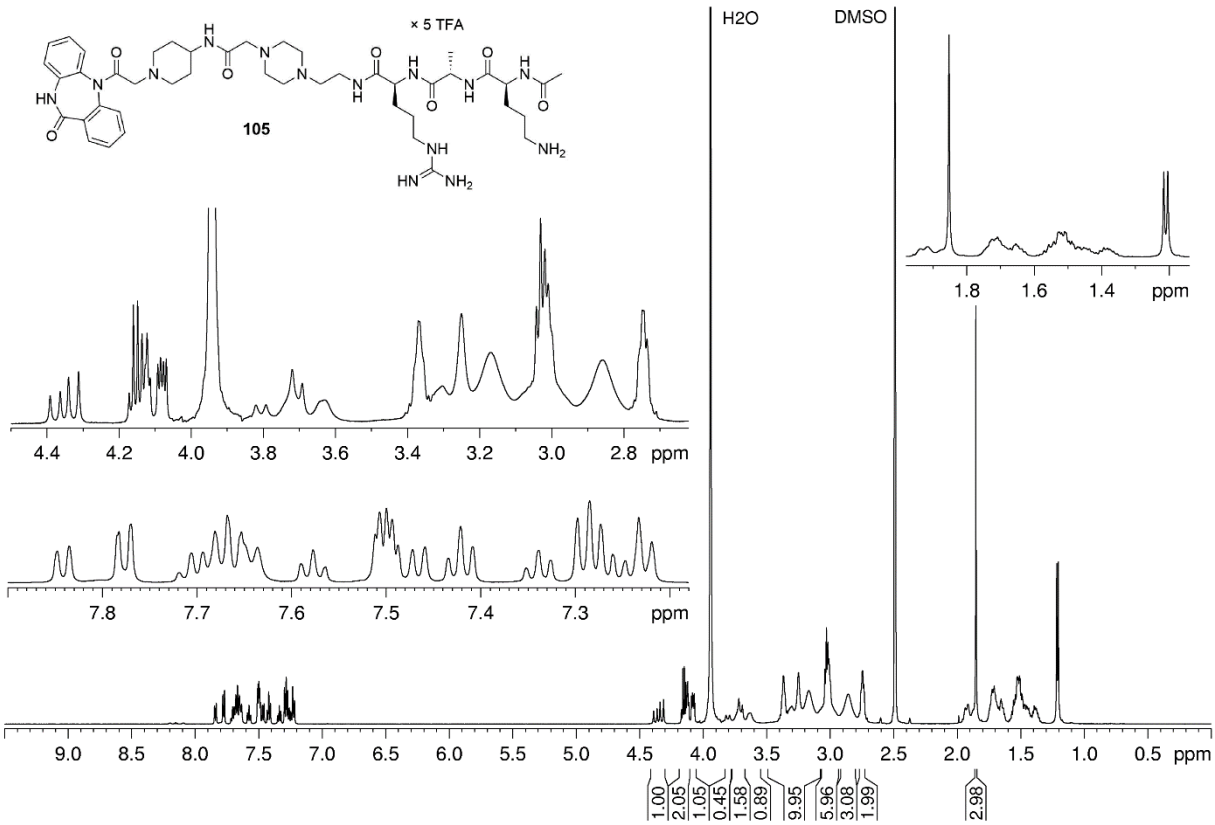
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **104**



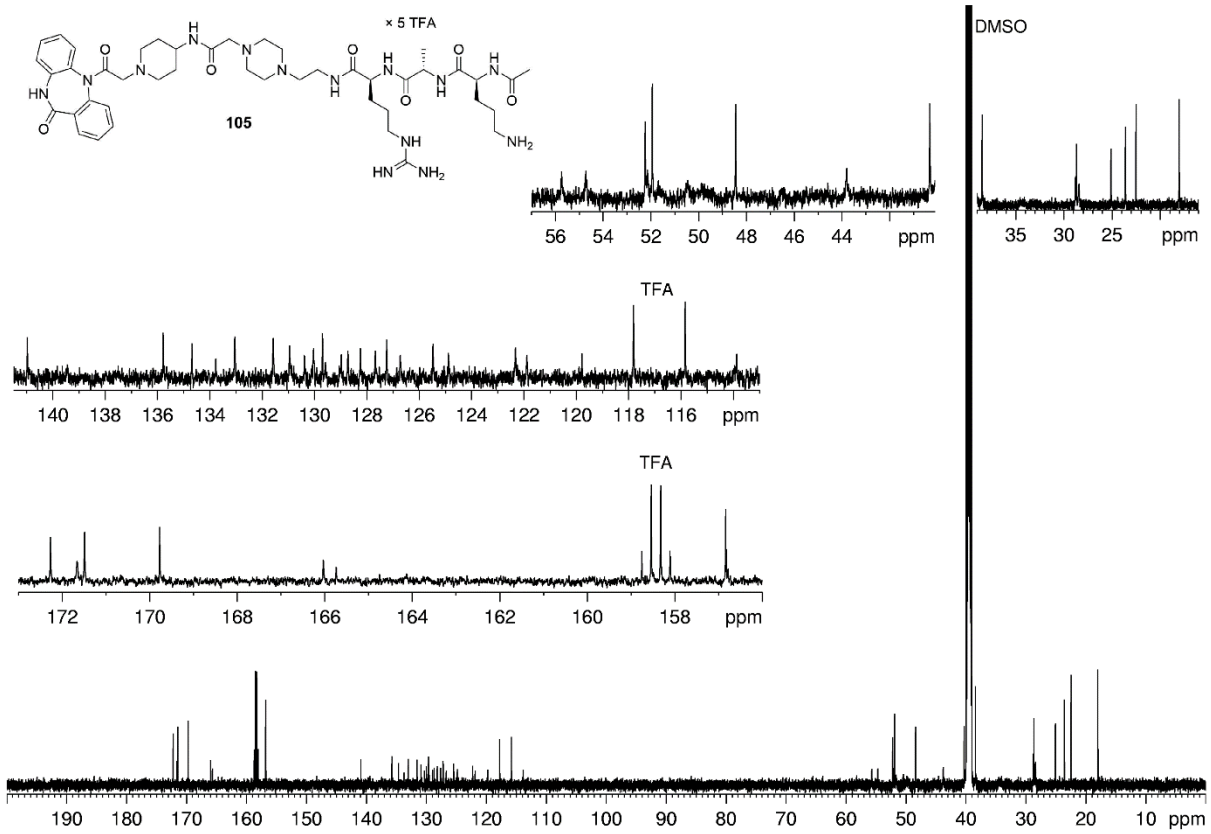
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **104**



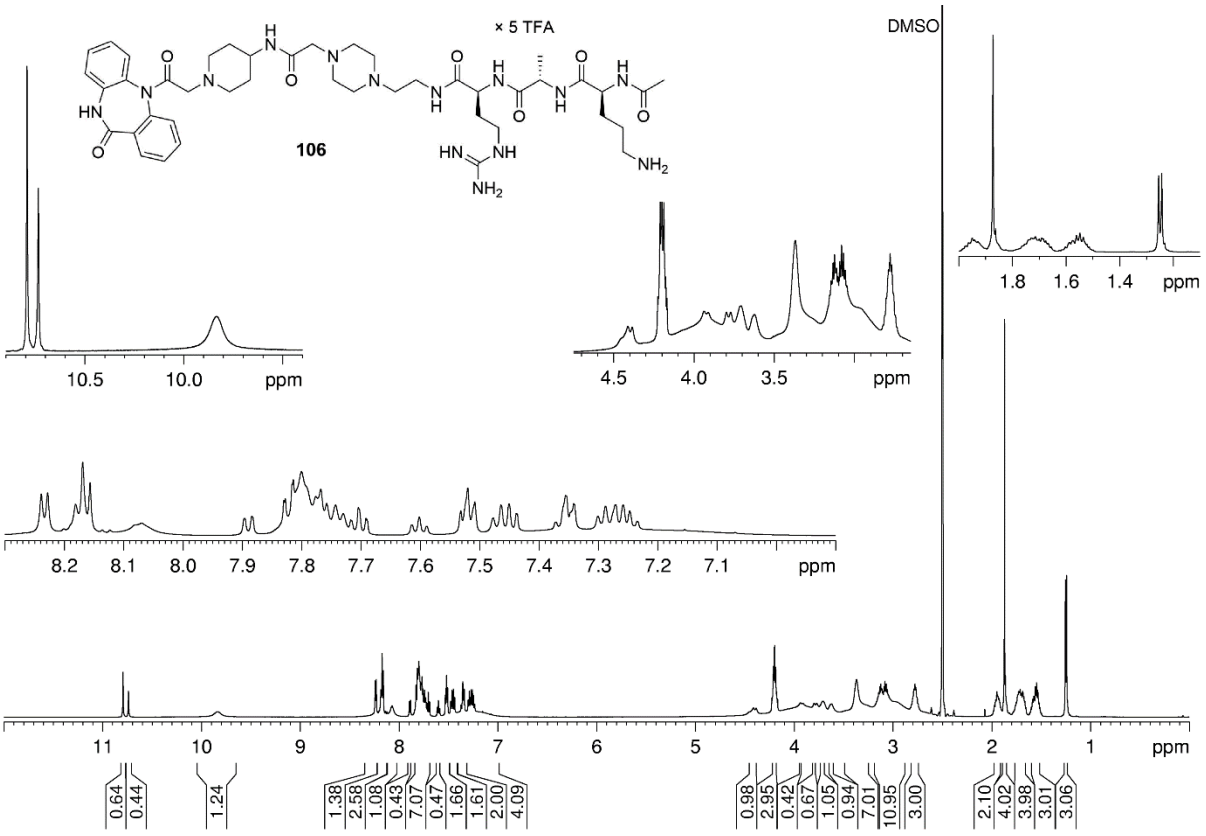
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **105**



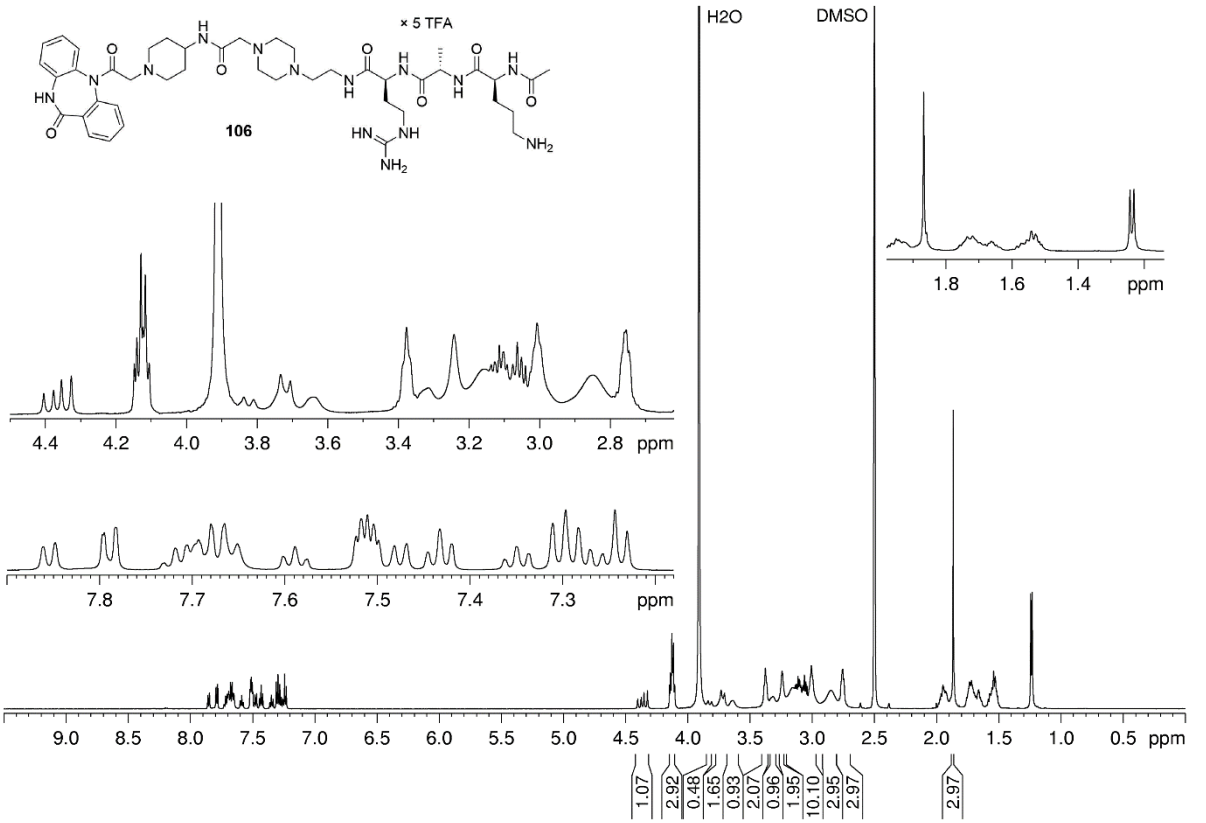
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **105**



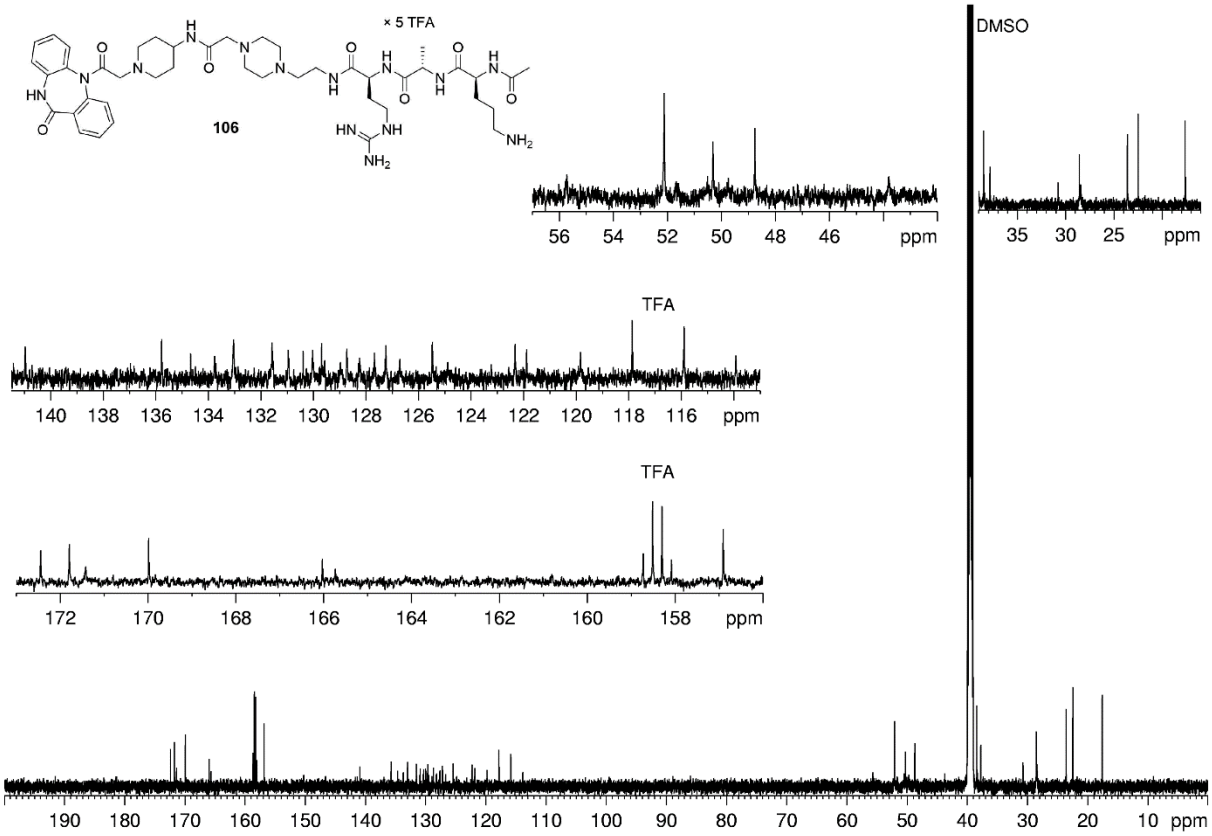
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **105**



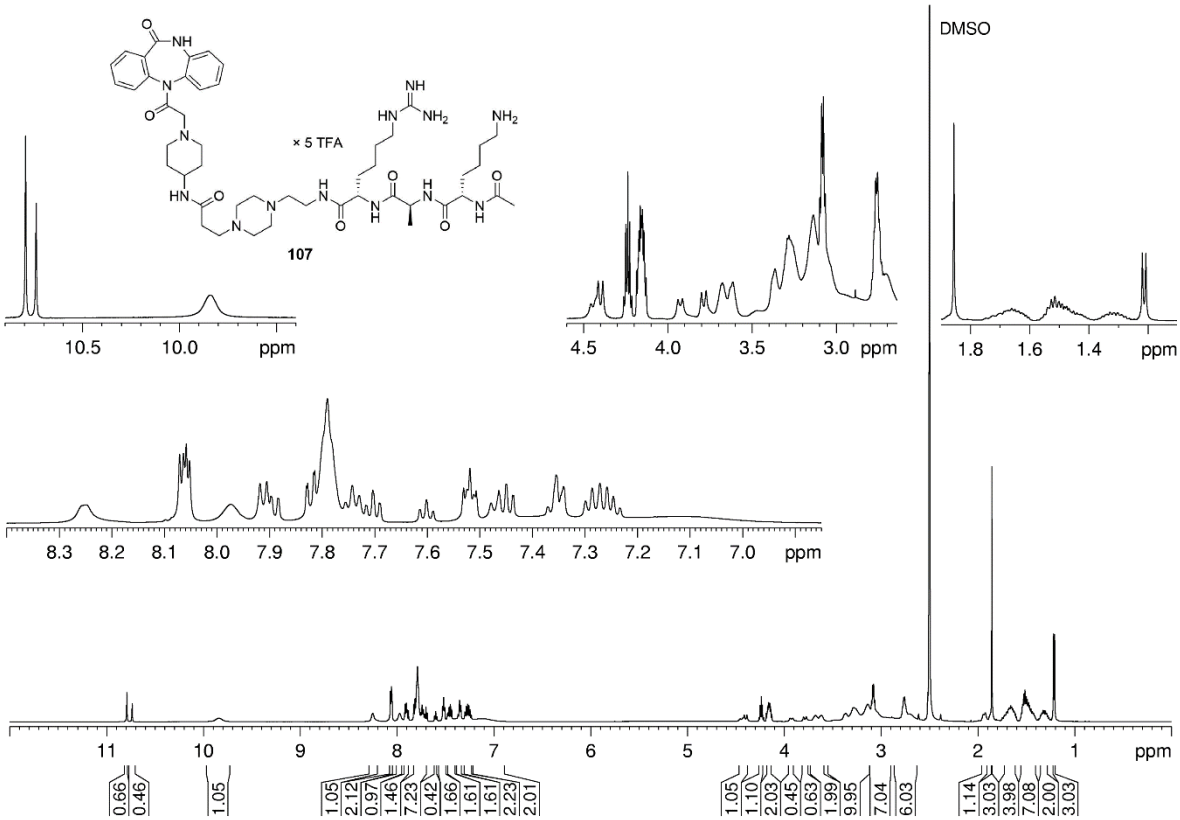
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **106**



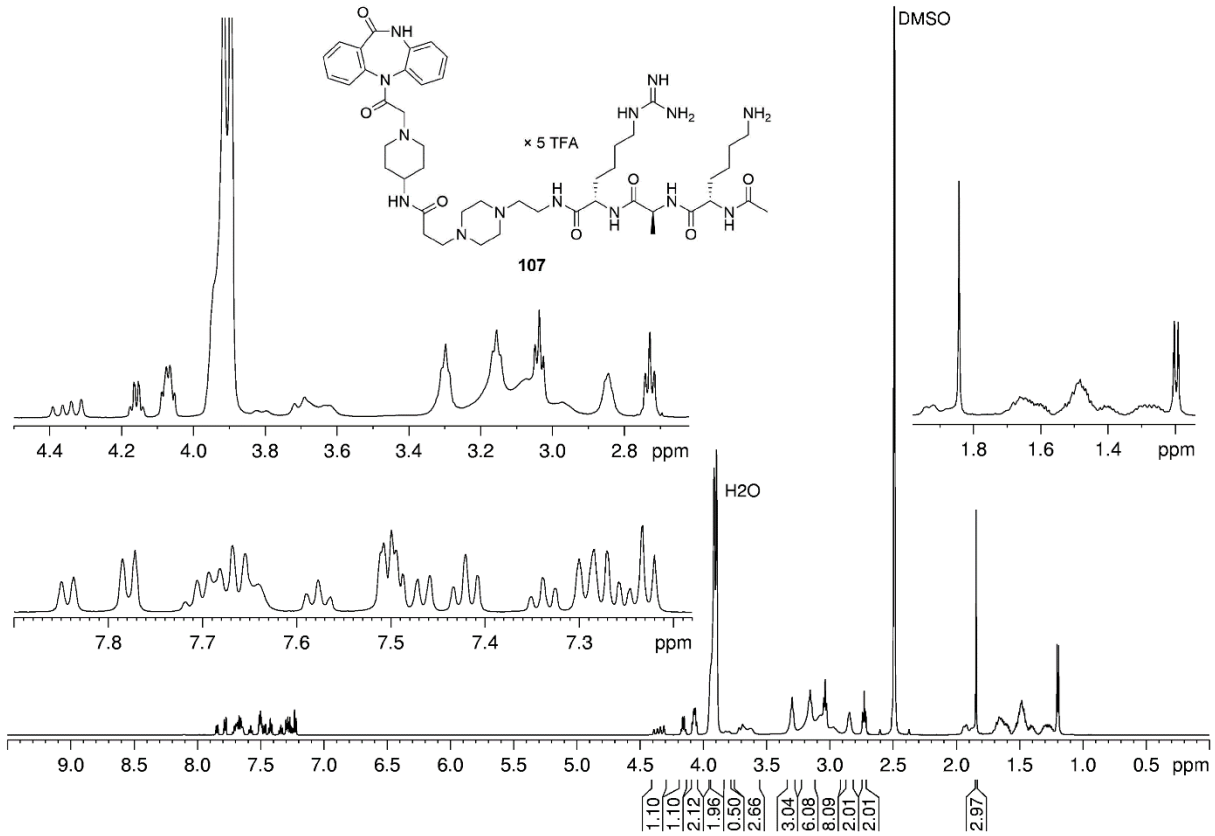
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **106**



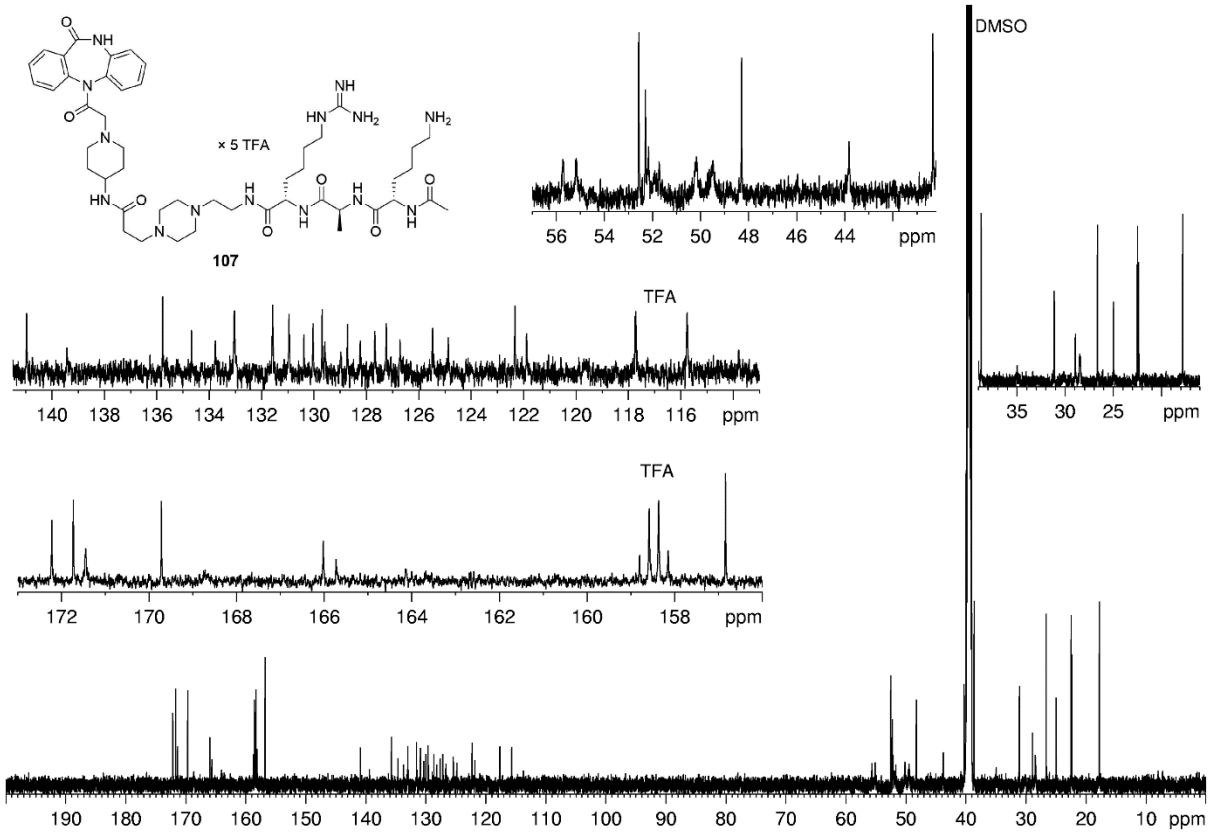
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **106**



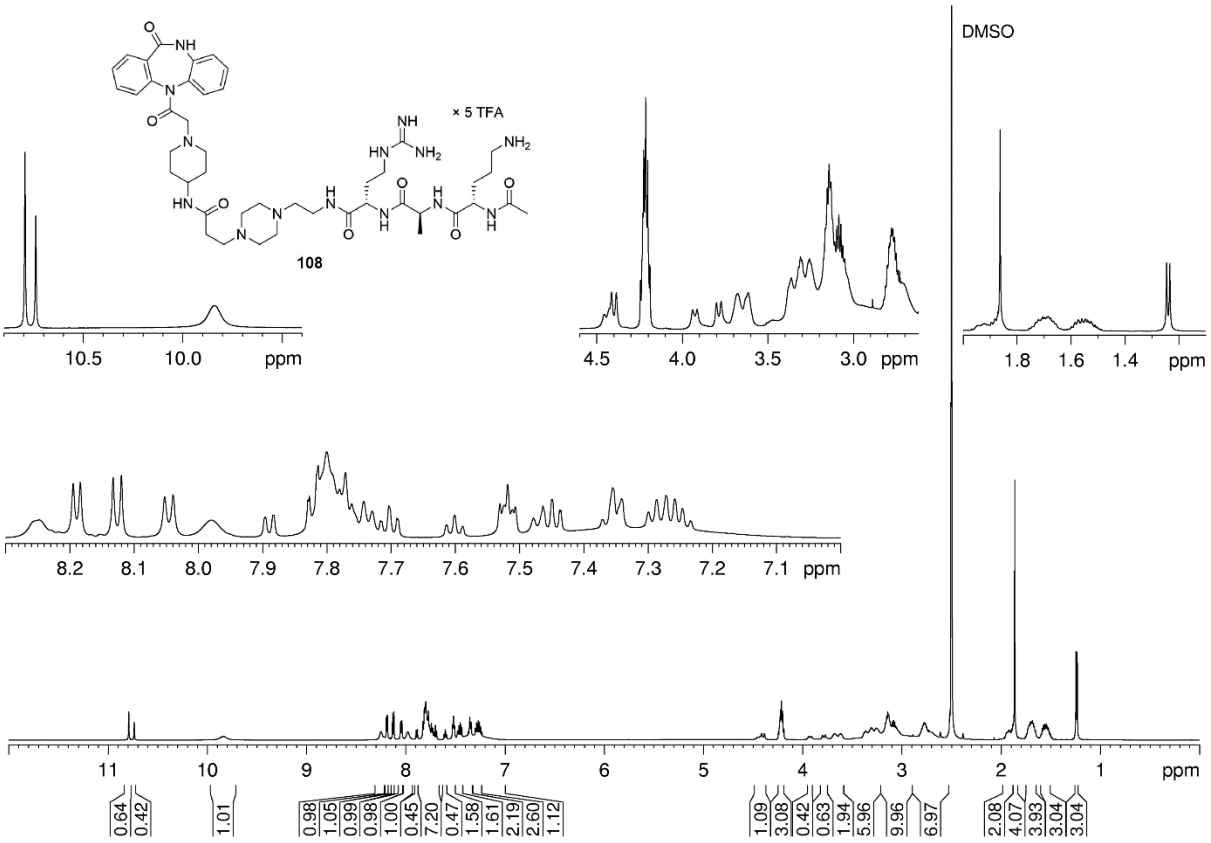
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **107**



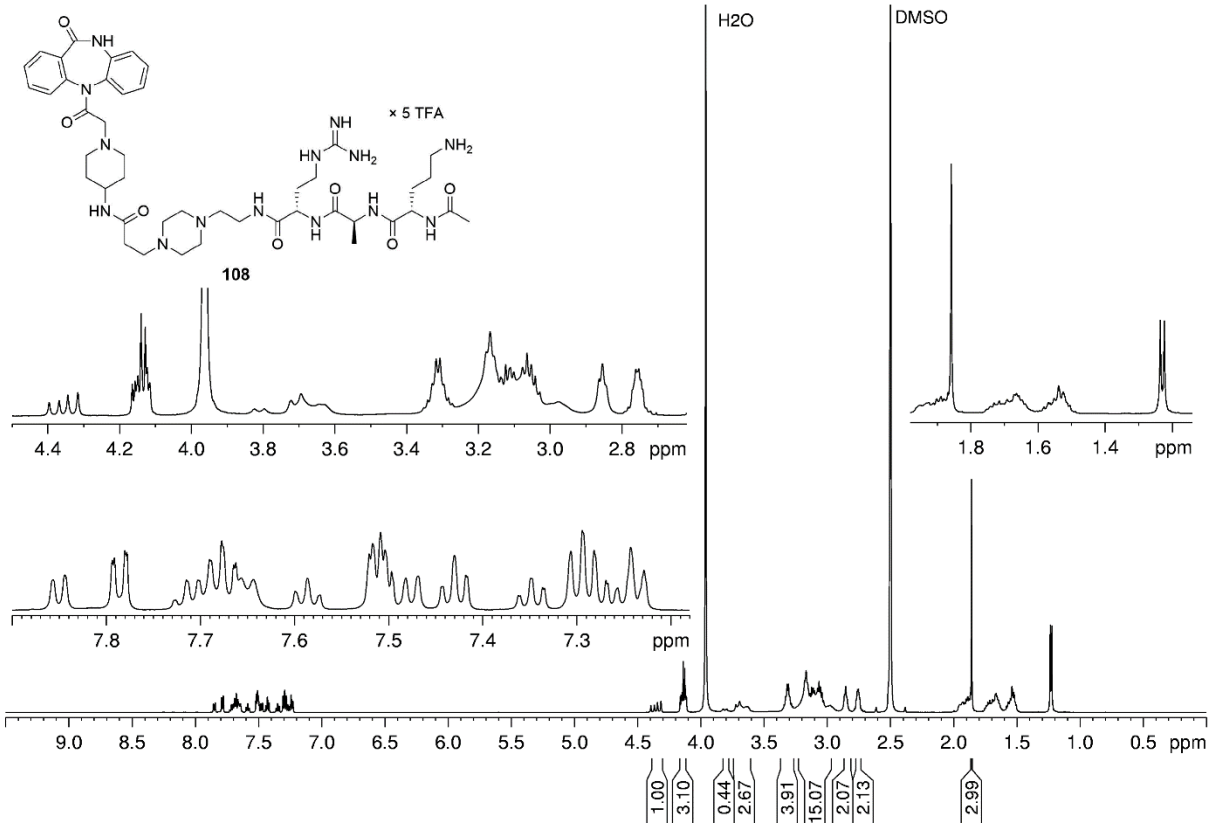
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **107**



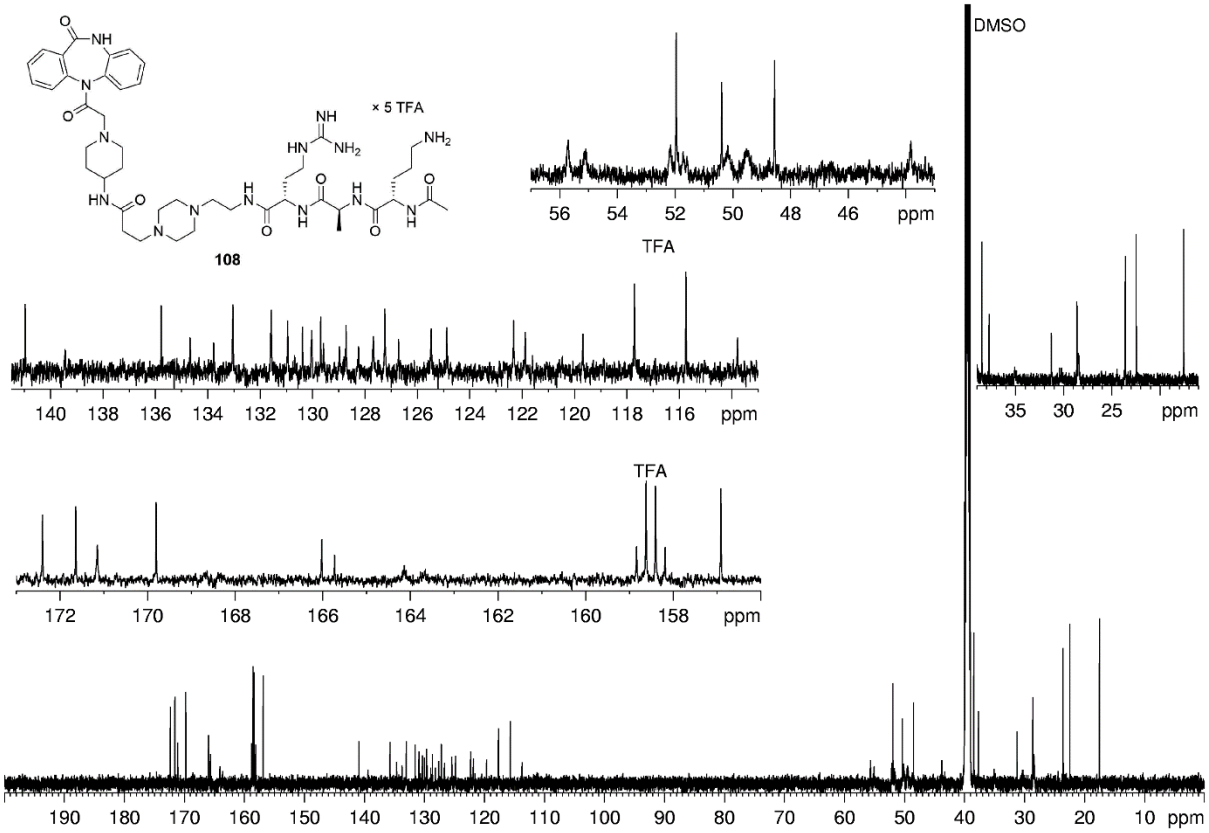
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **107**



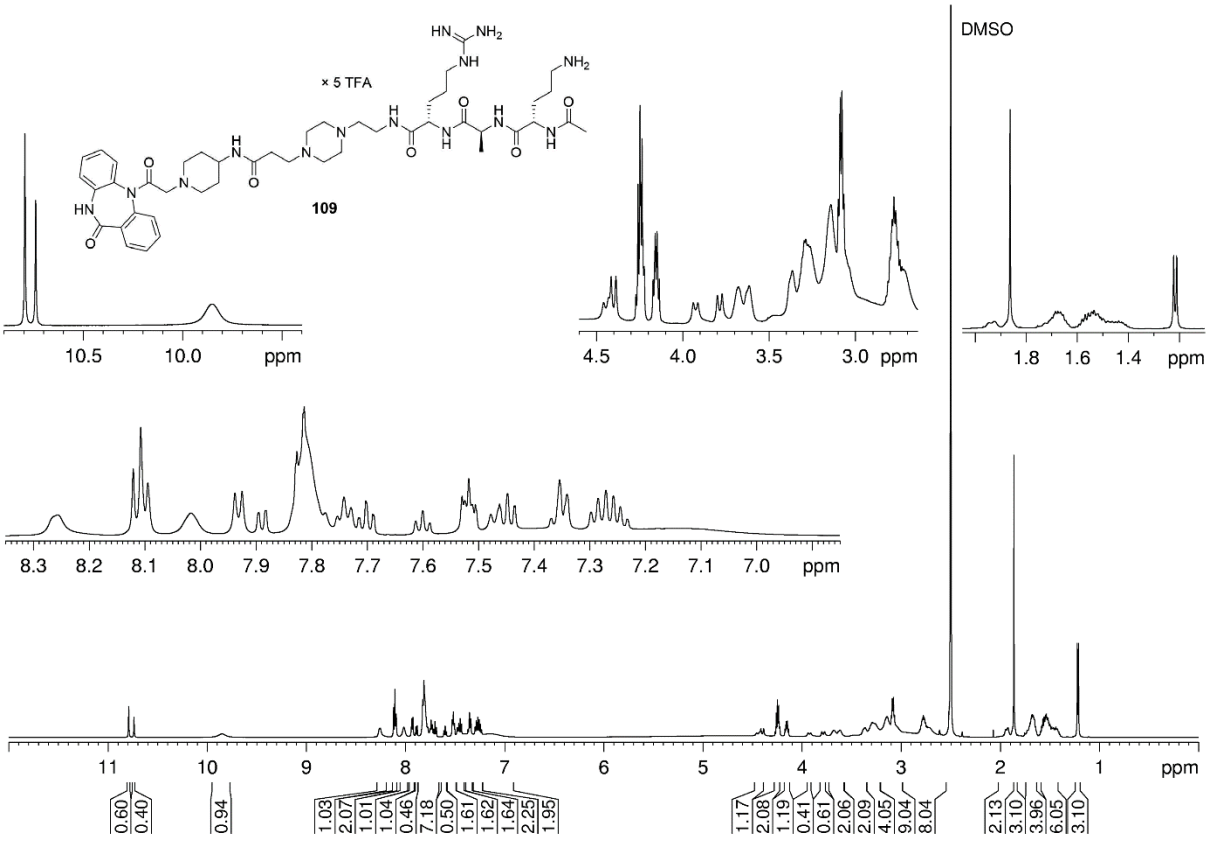
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **108**



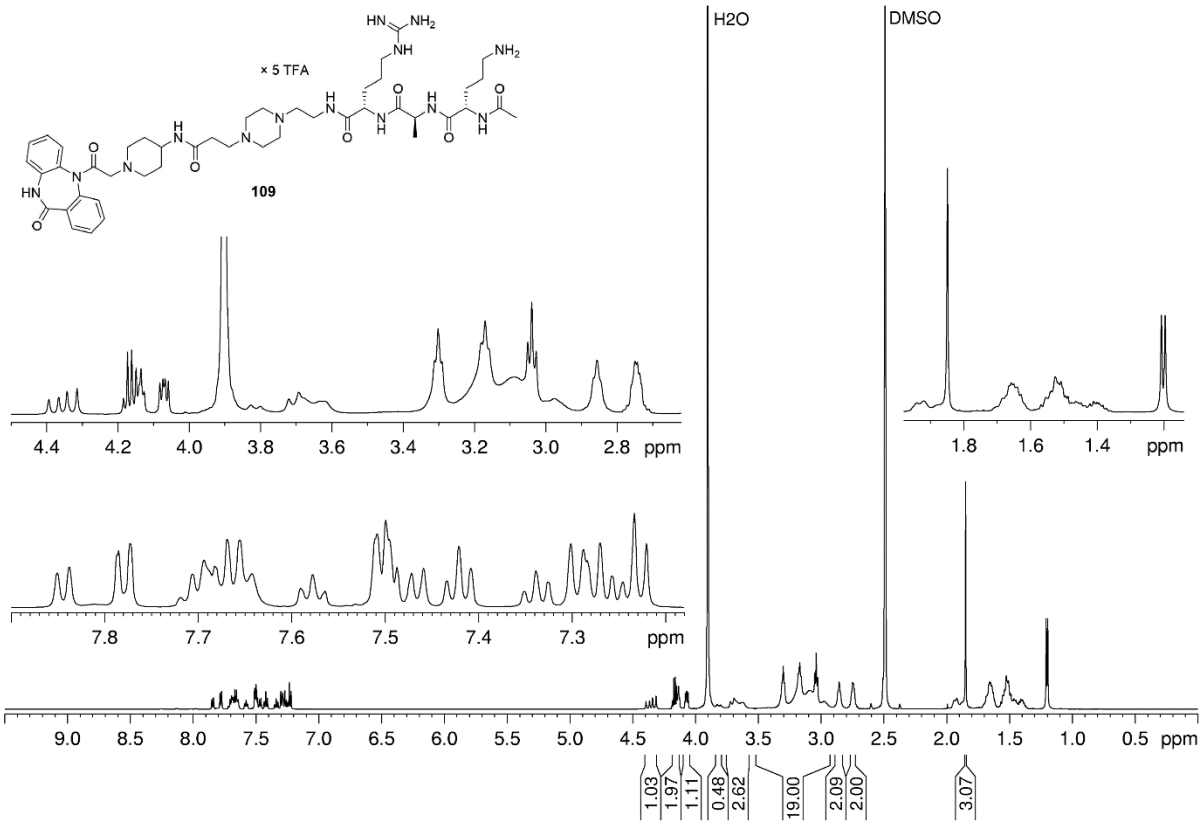
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **108**



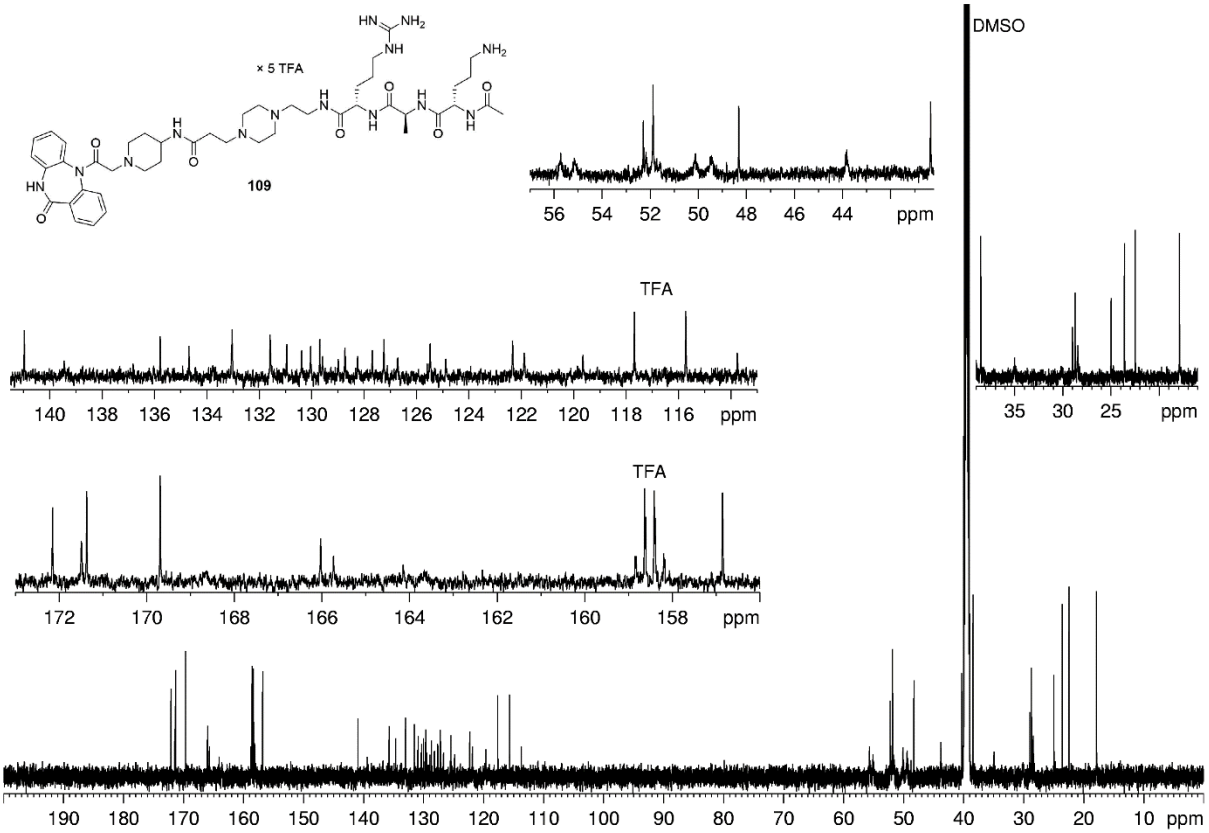
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **108**



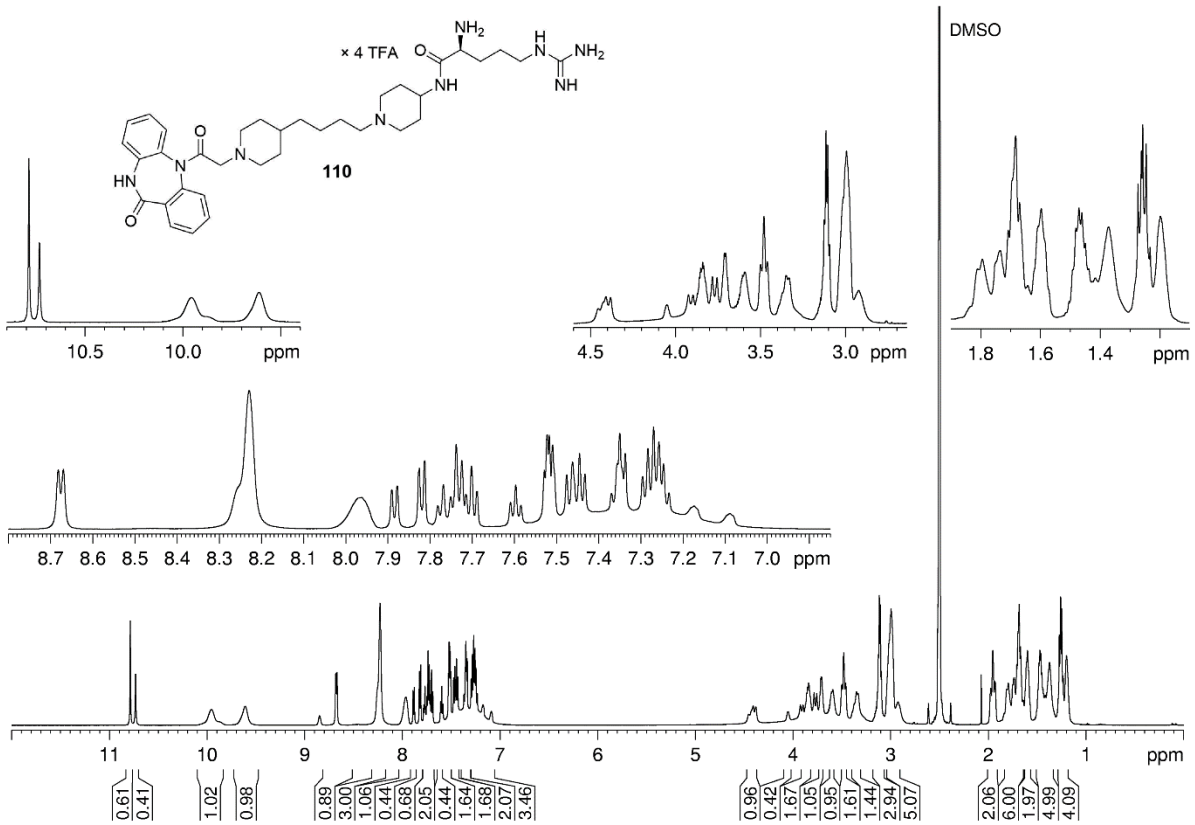
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **109**



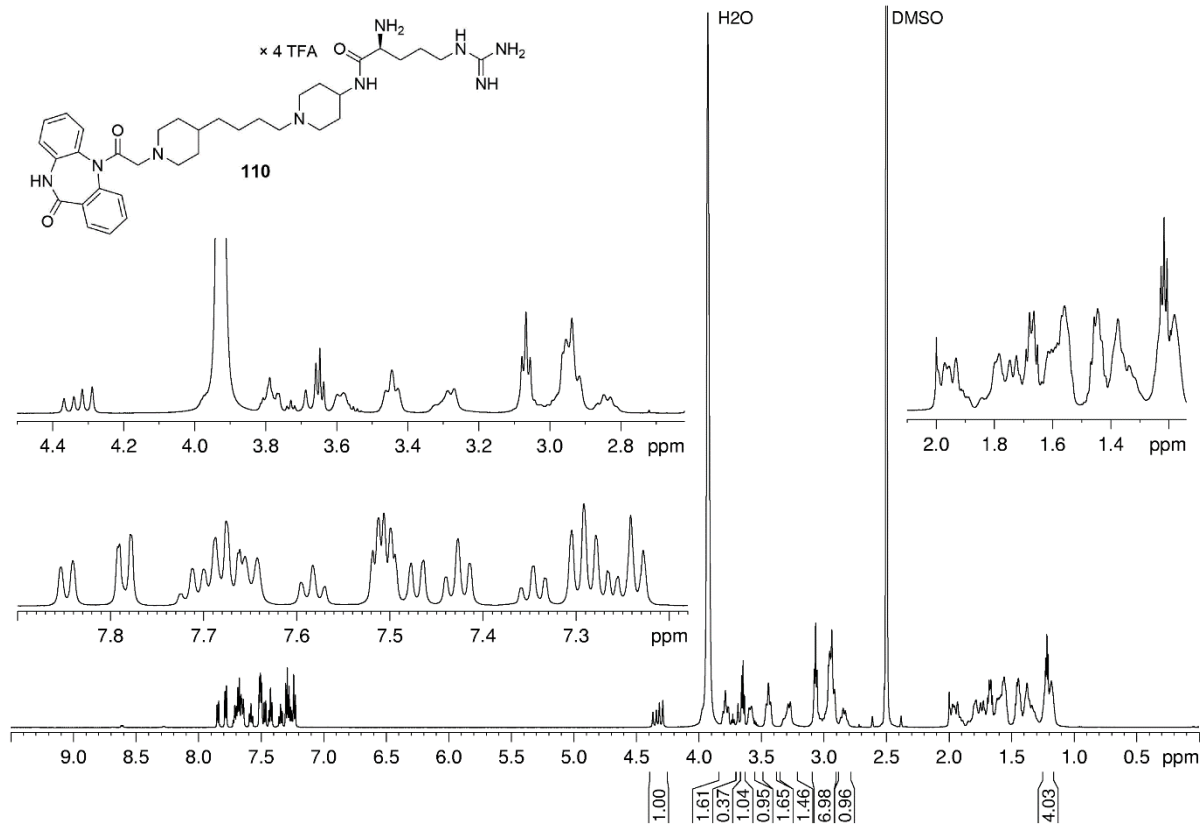
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **109**



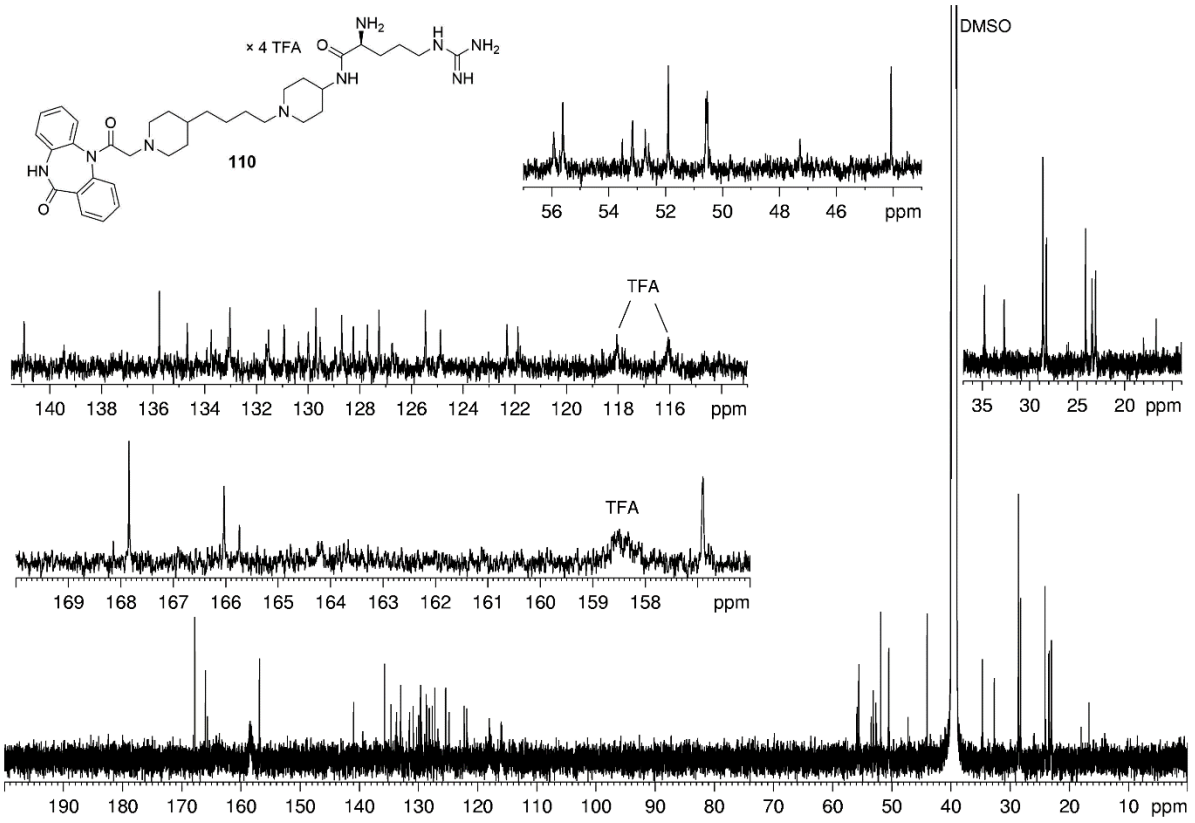
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **109**



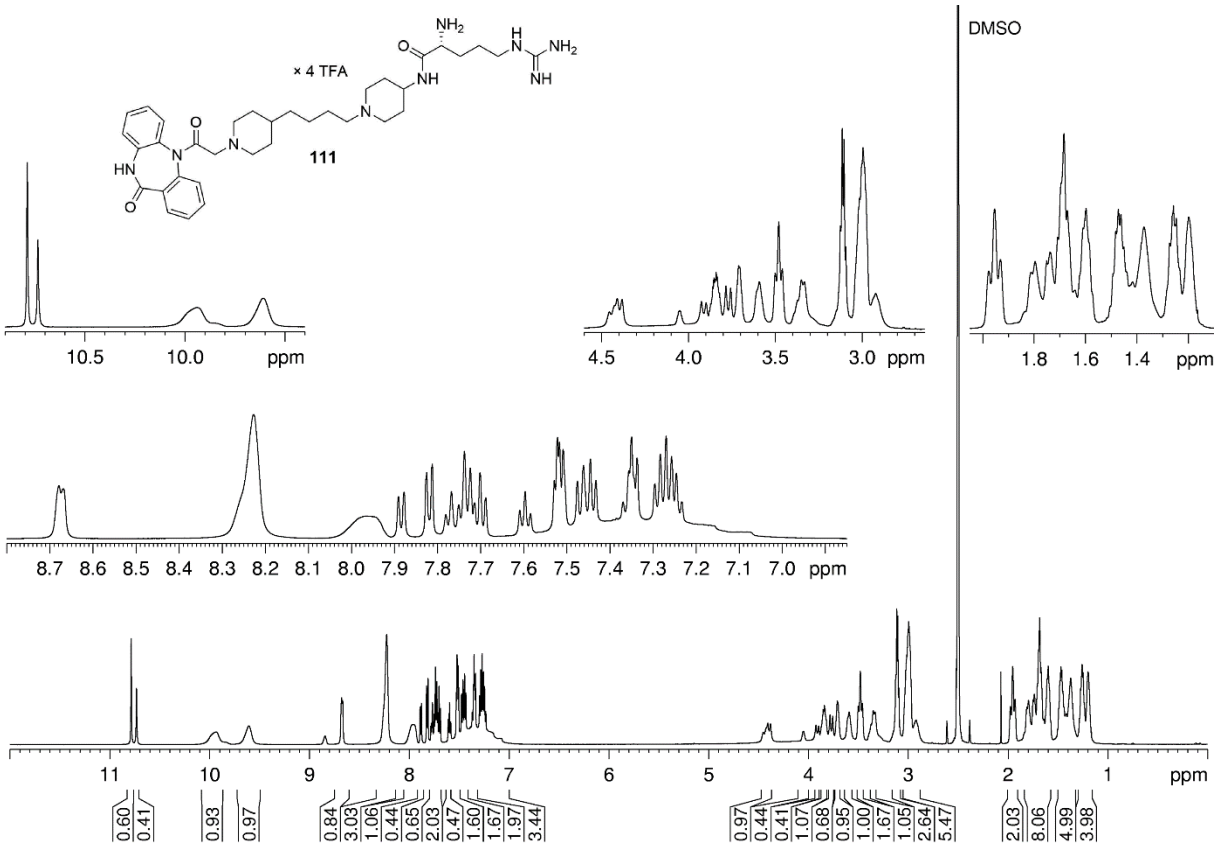
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **110**



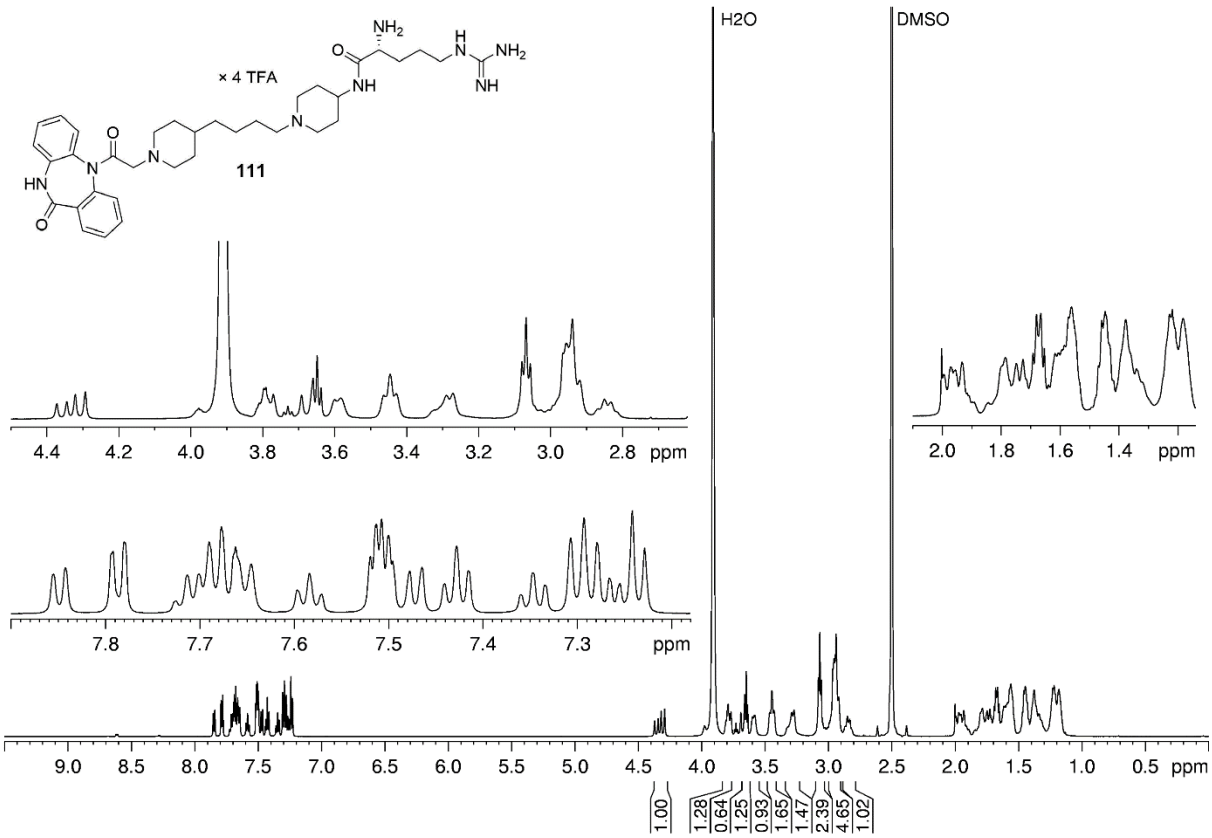
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **110**



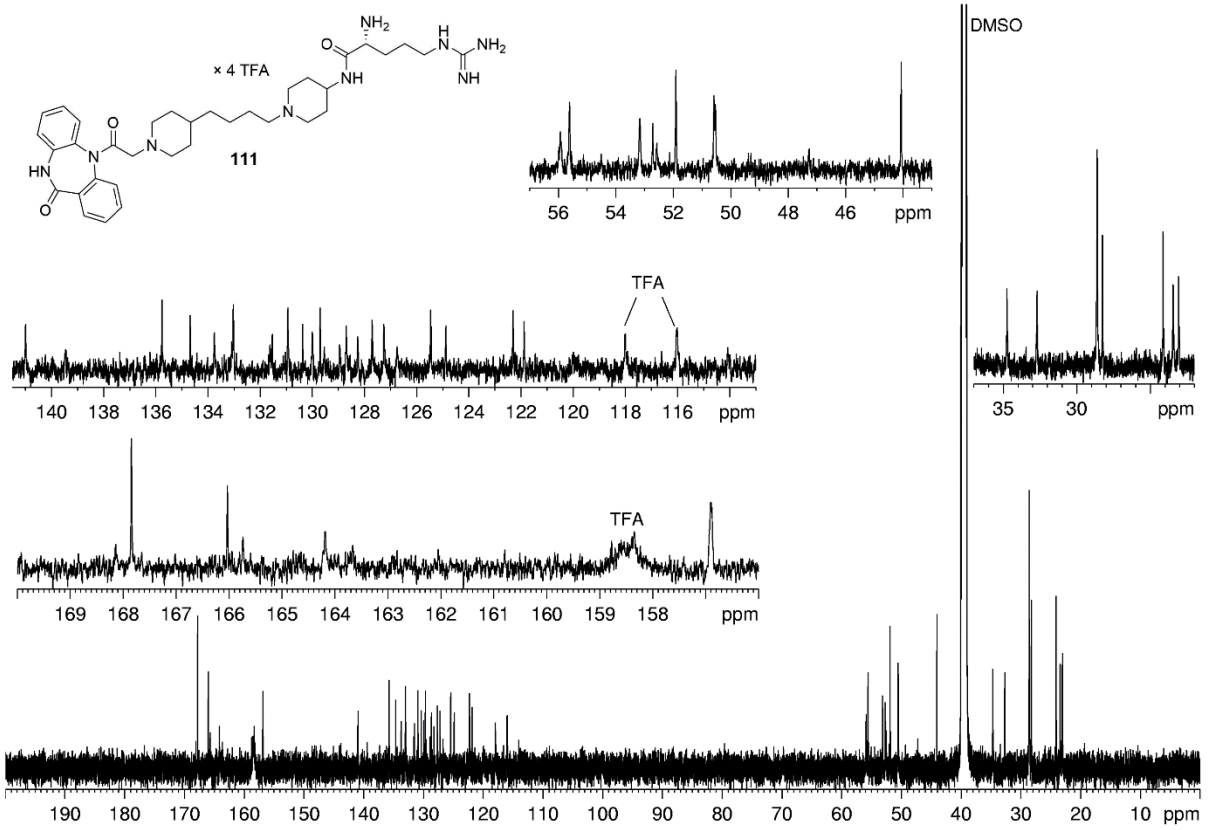
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **110**



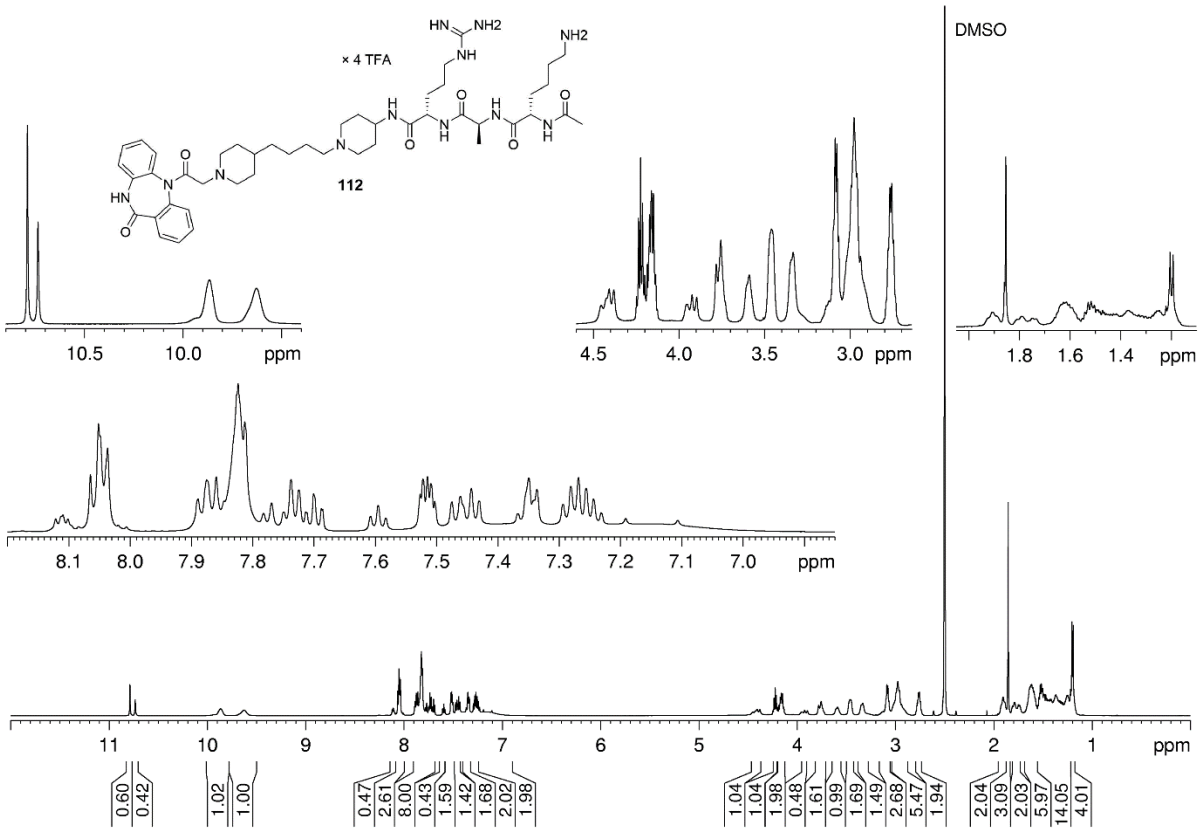
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **111**



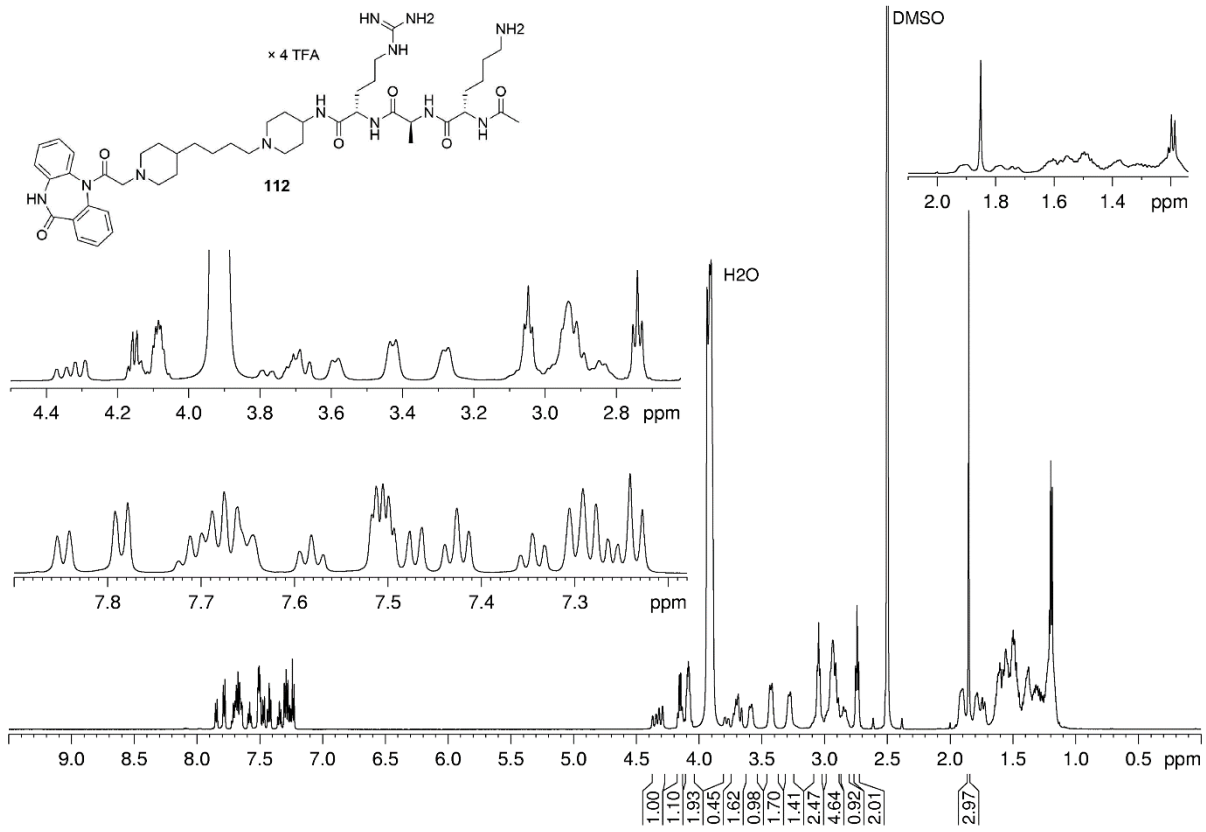
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **111**



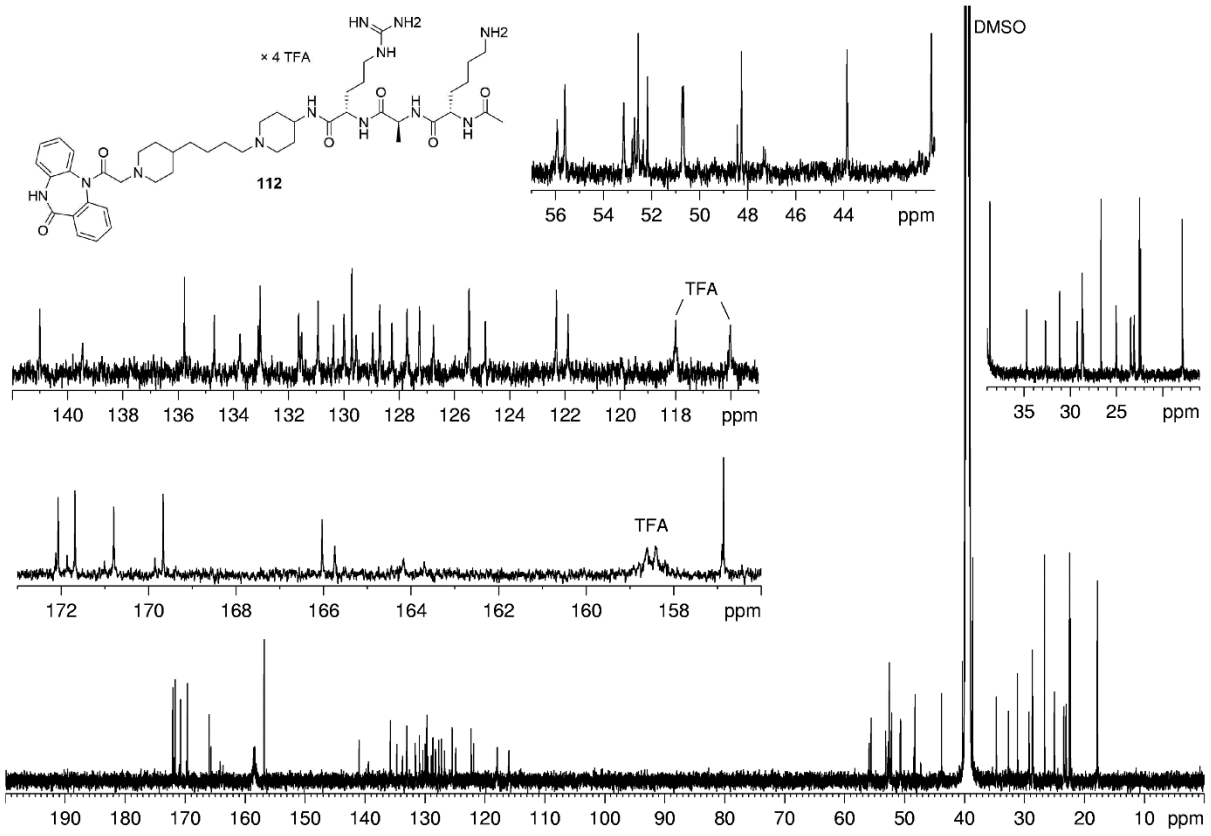
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **111**



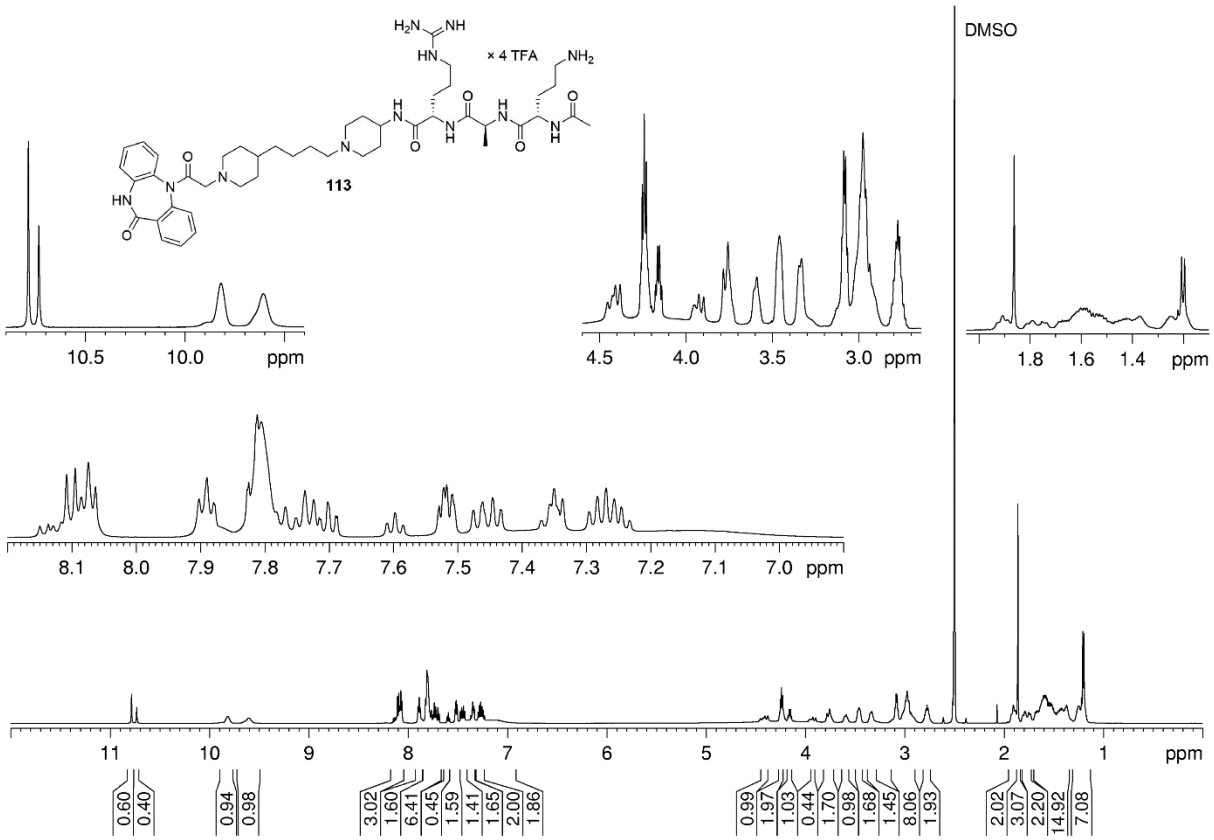
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **112**



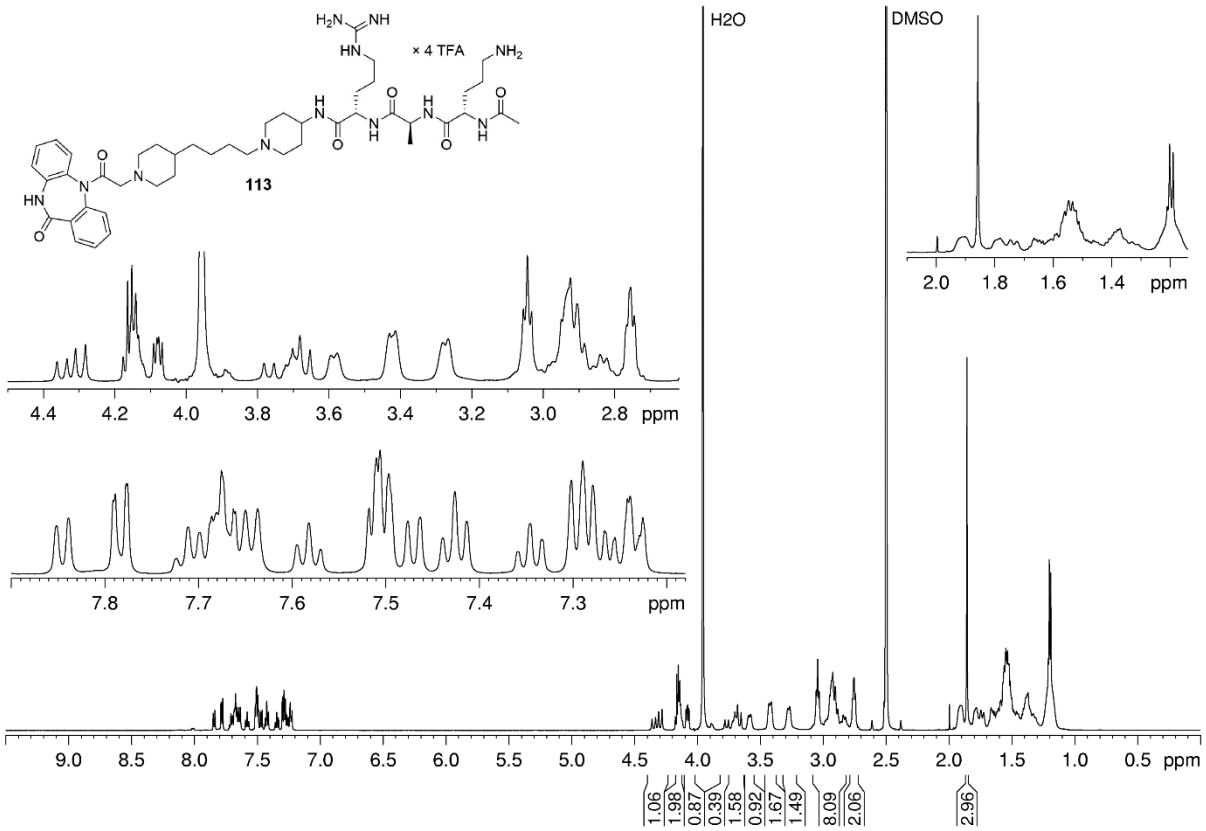
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **112**



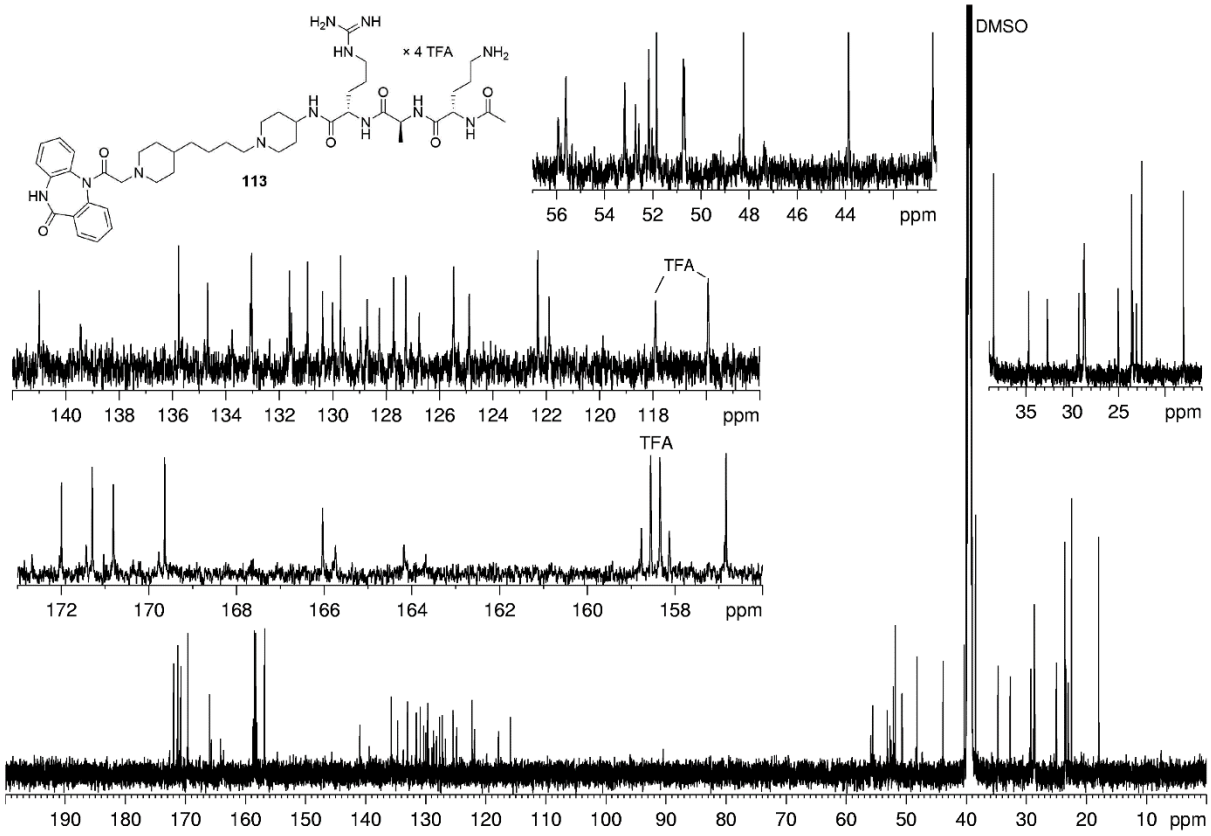
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **112**



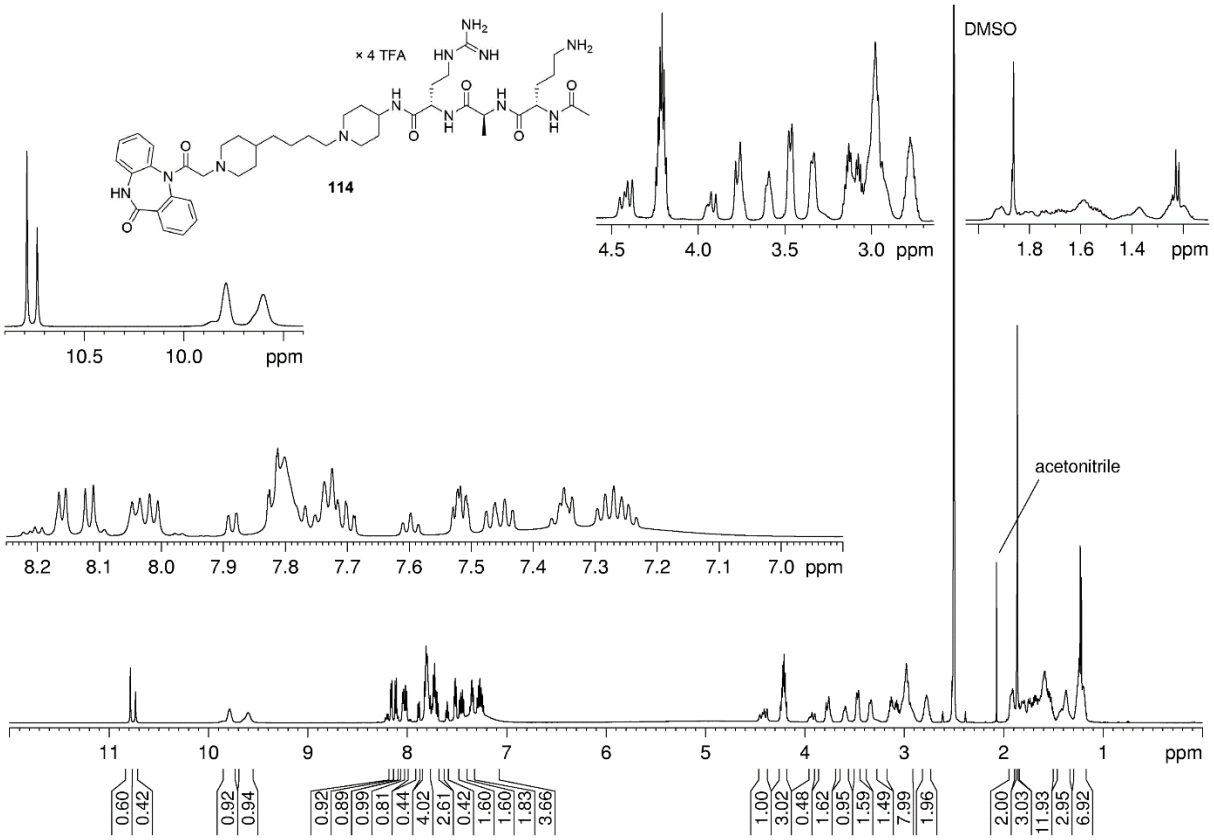
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **113**



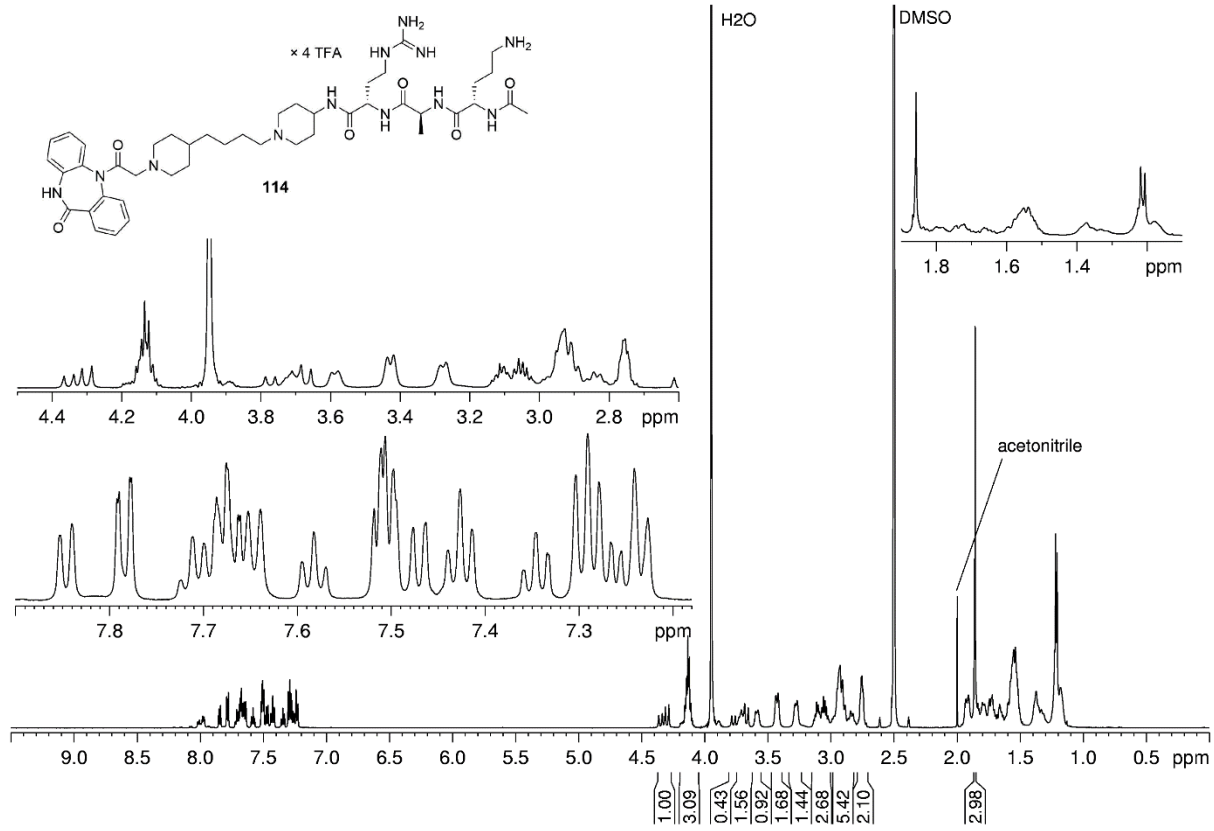
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **113**



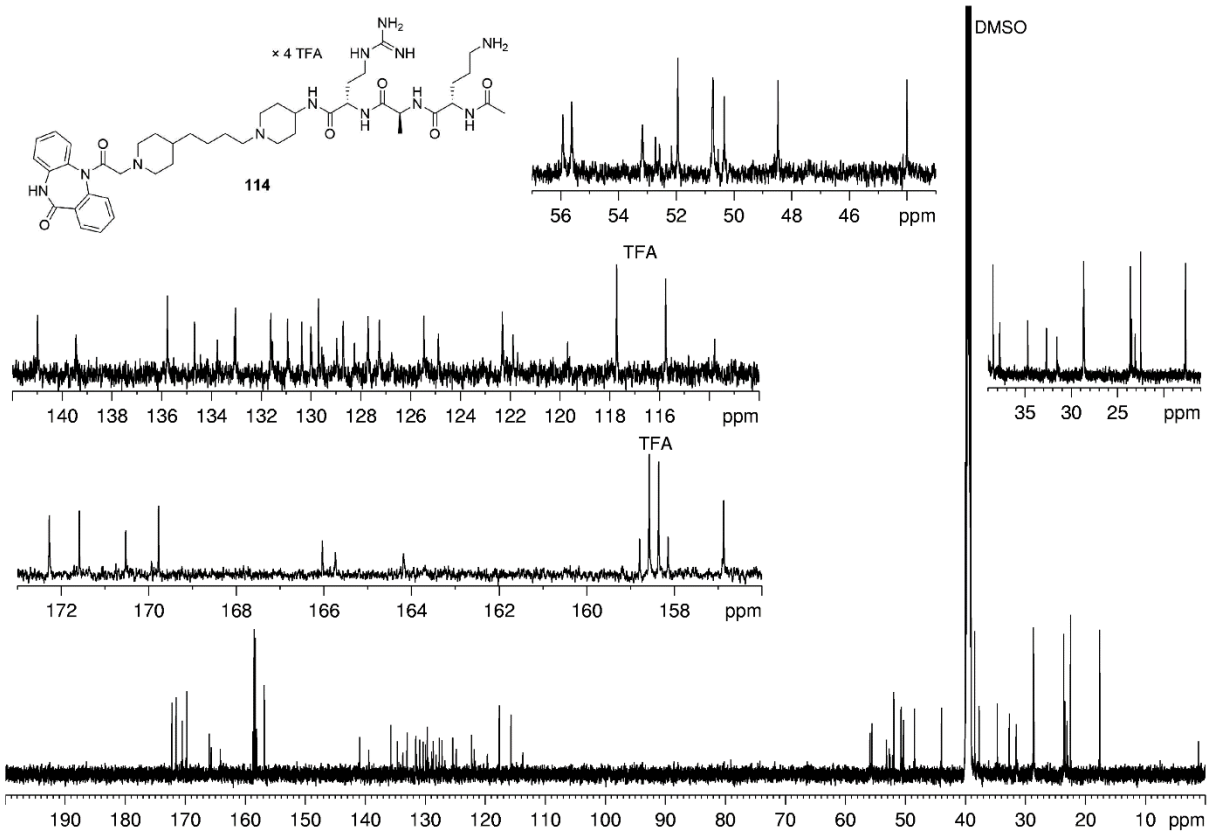
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **113**



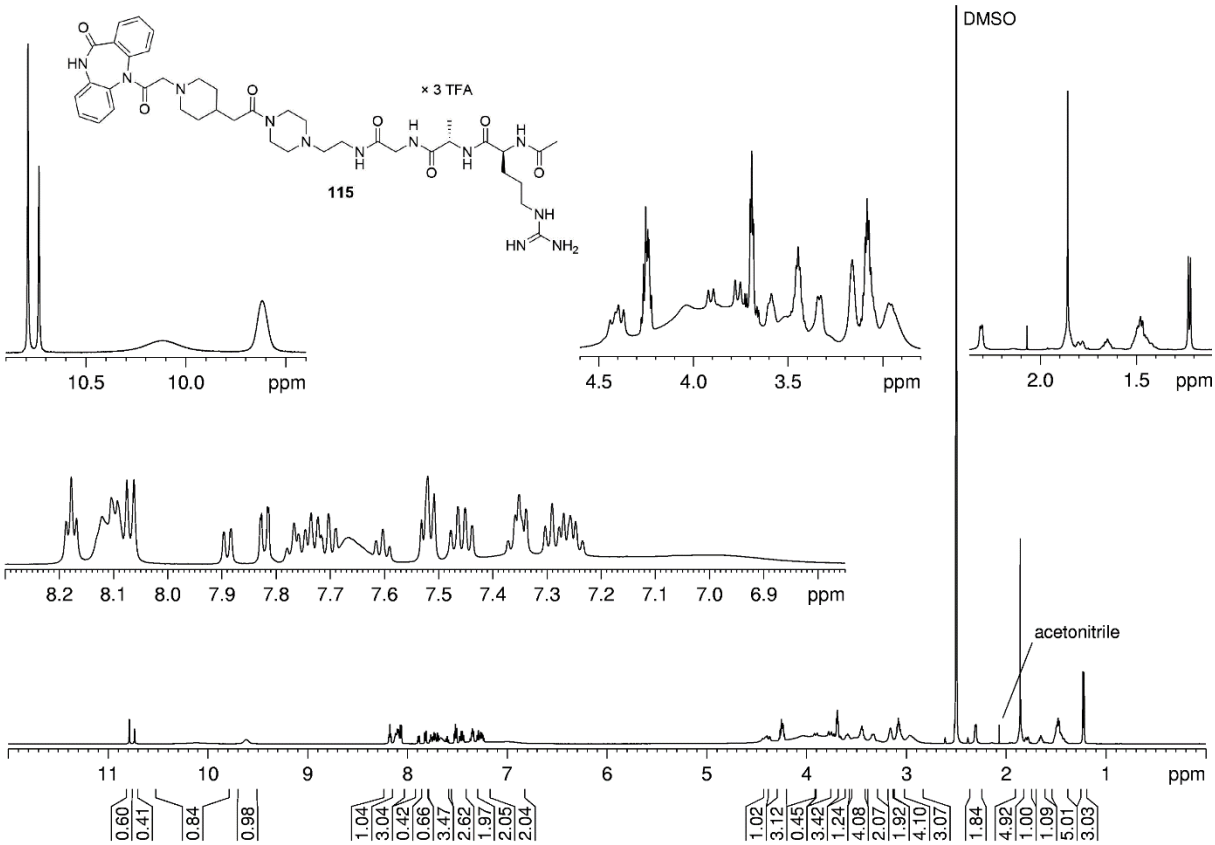
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **114**



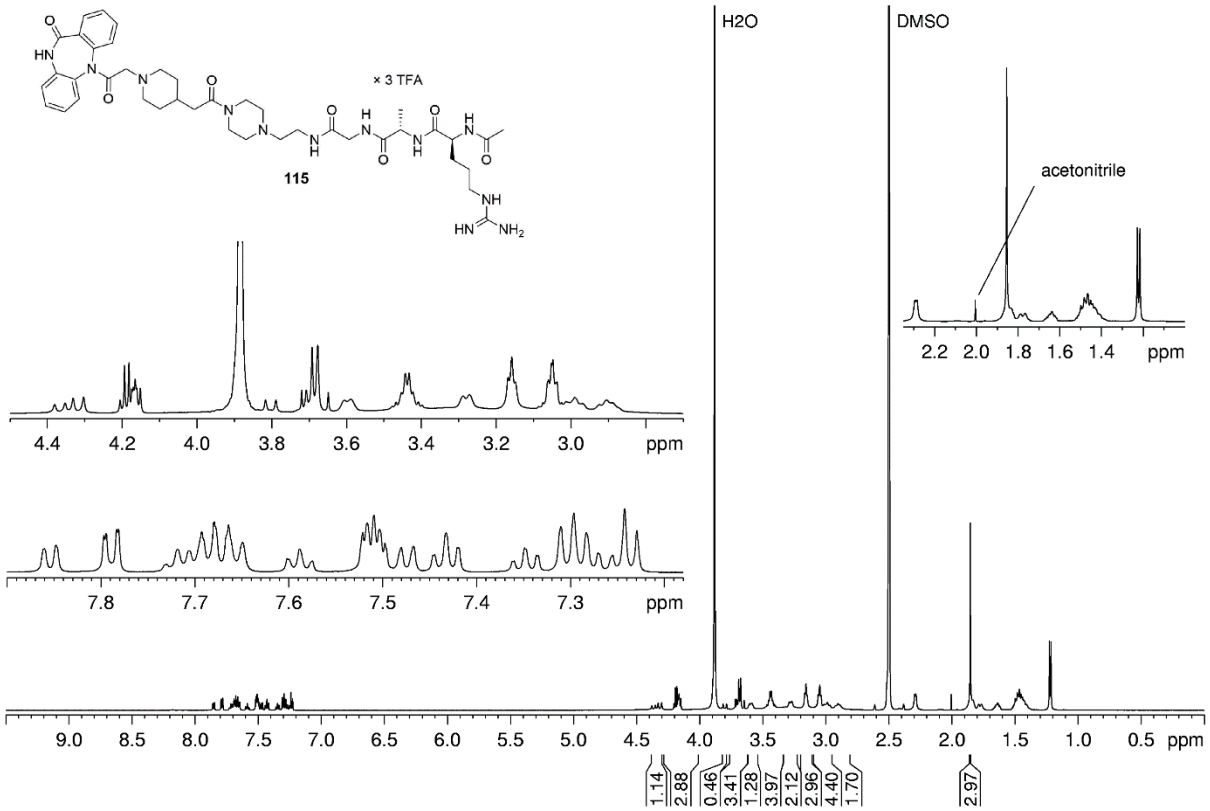
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **114**



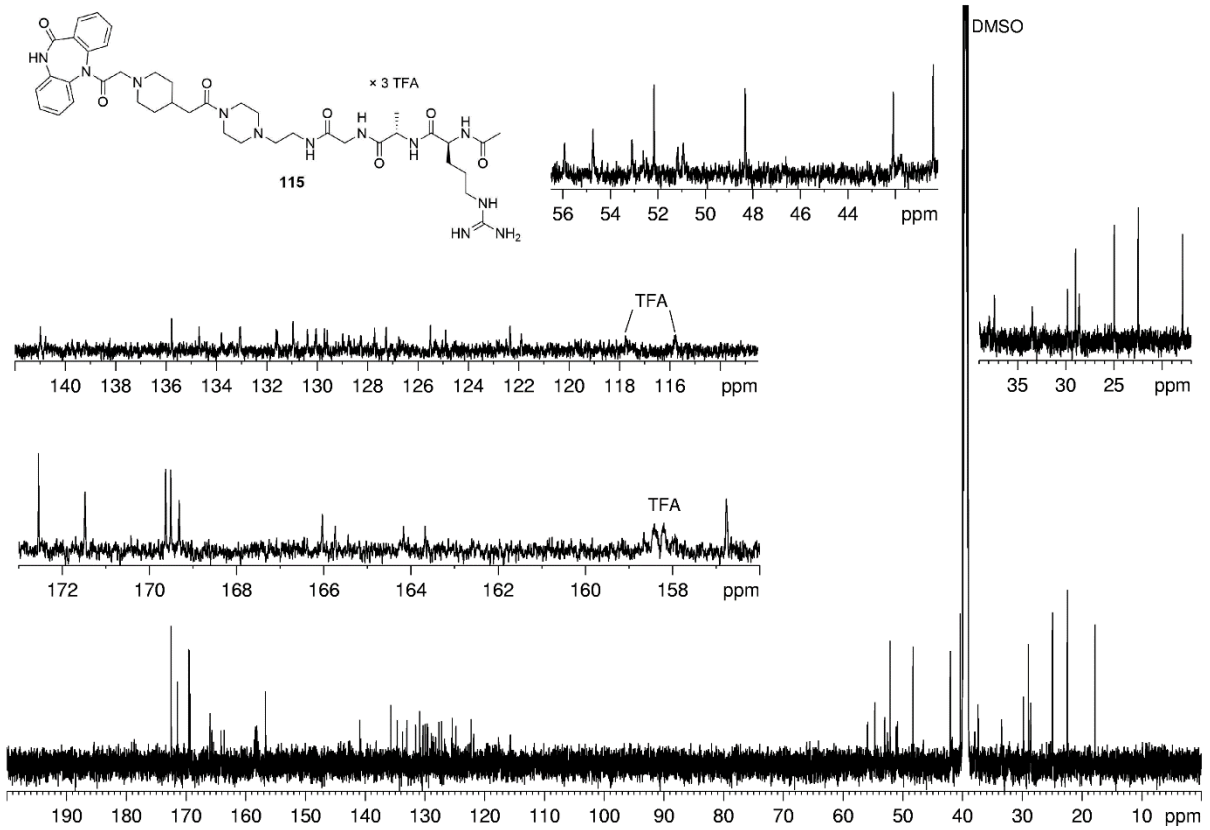
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **114**



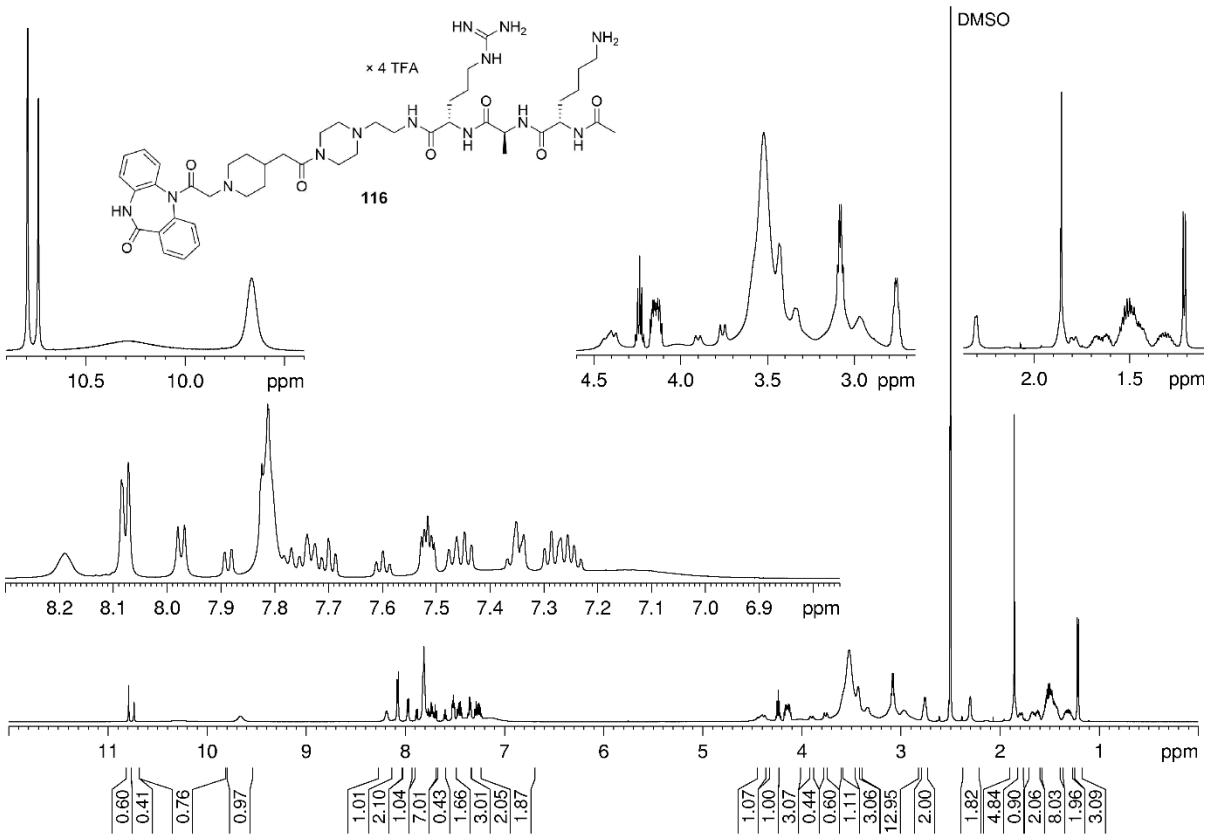
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **115**



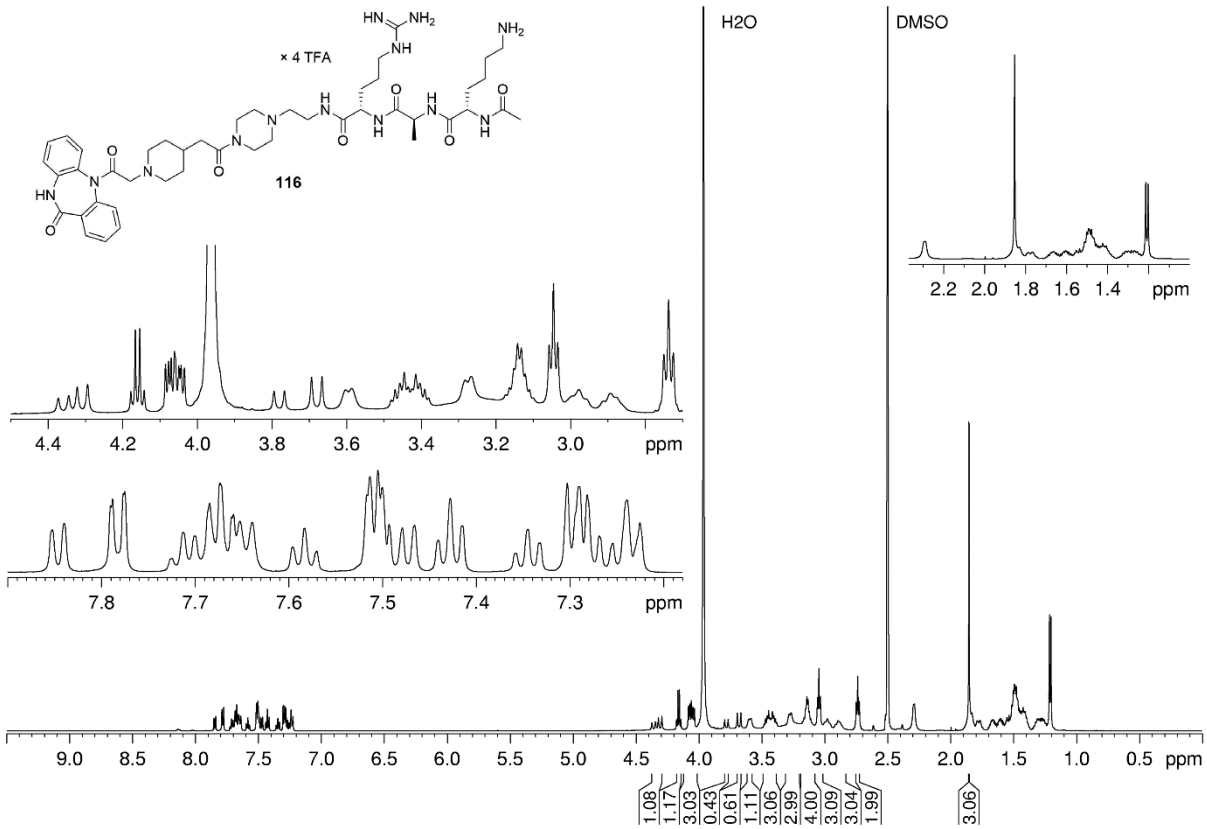
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **115**



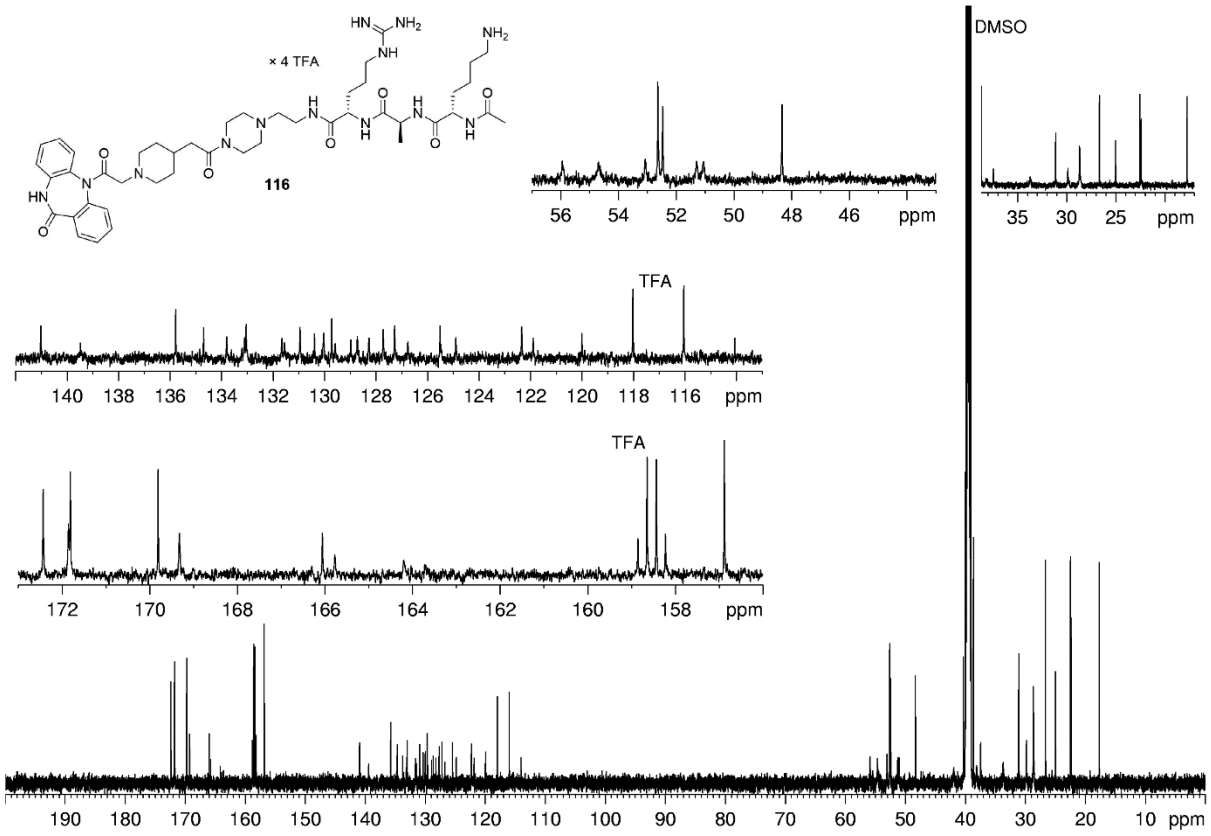
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **115**



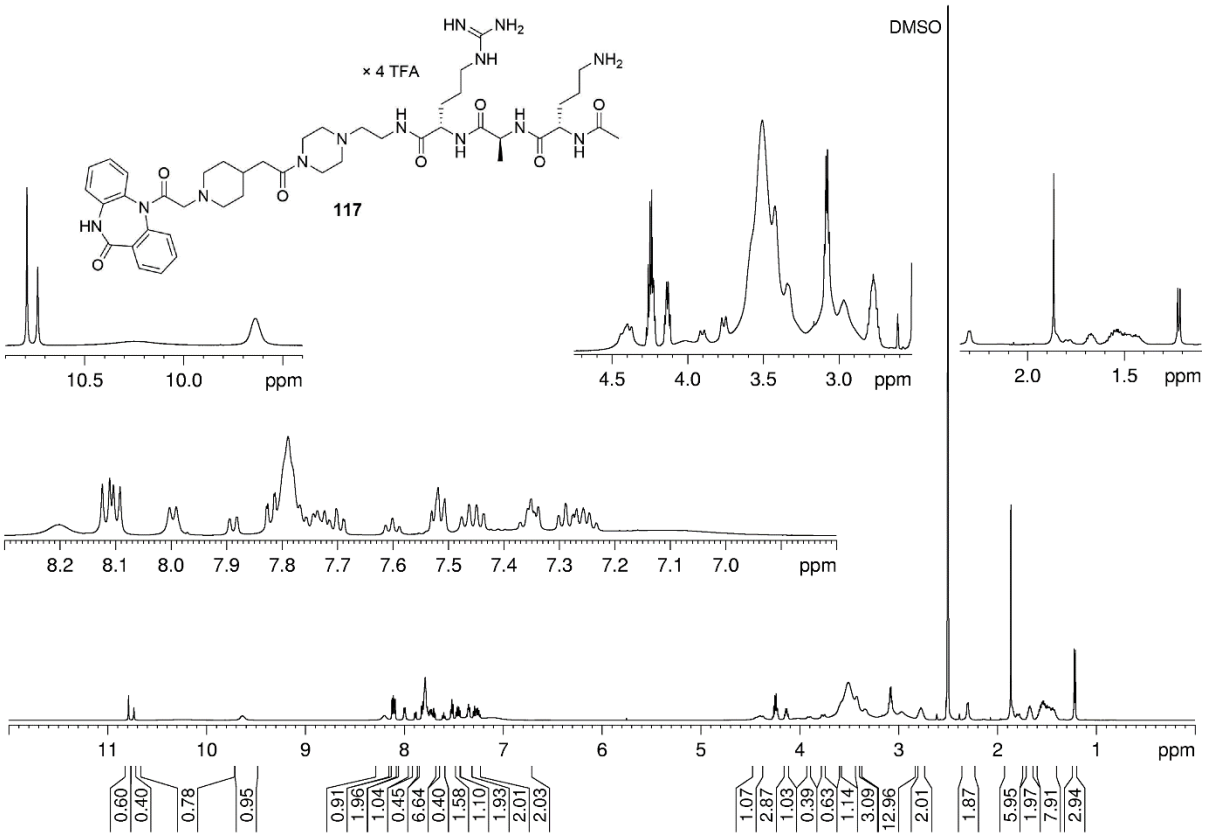
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **116**



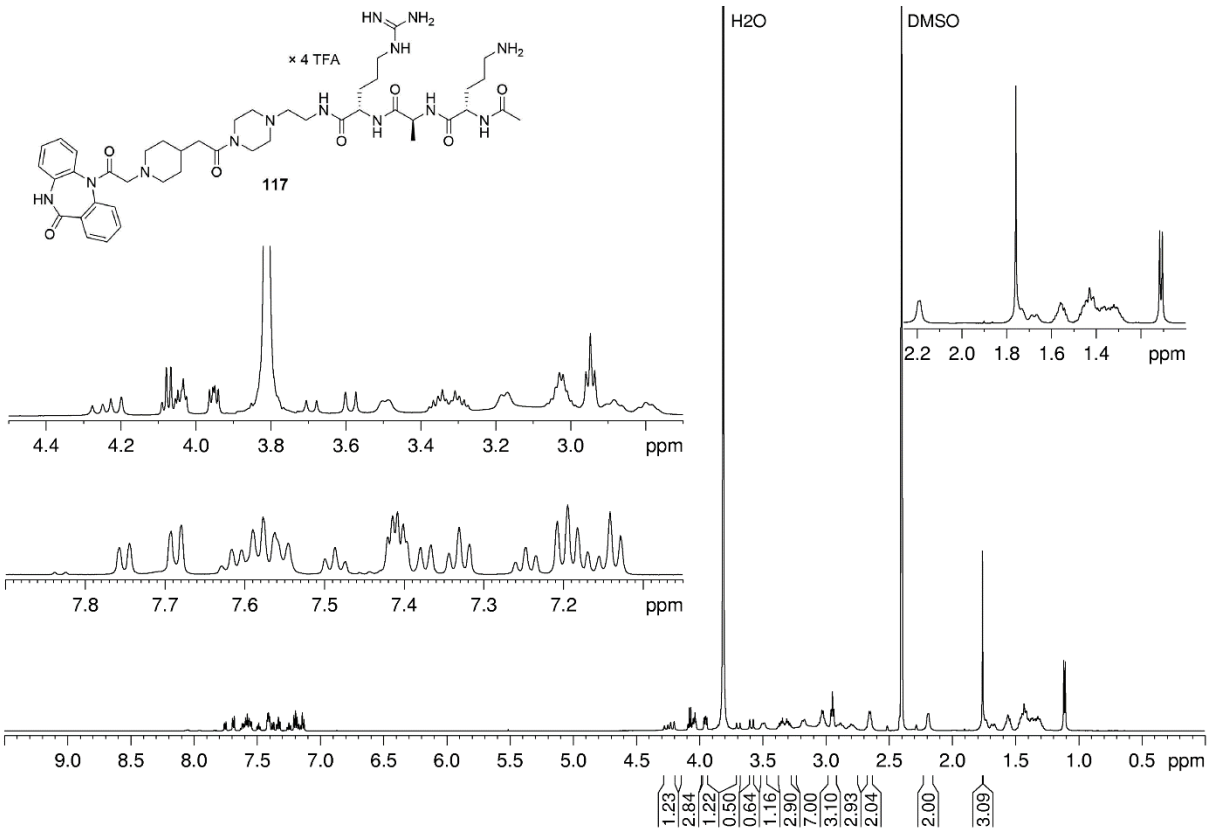
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **116**



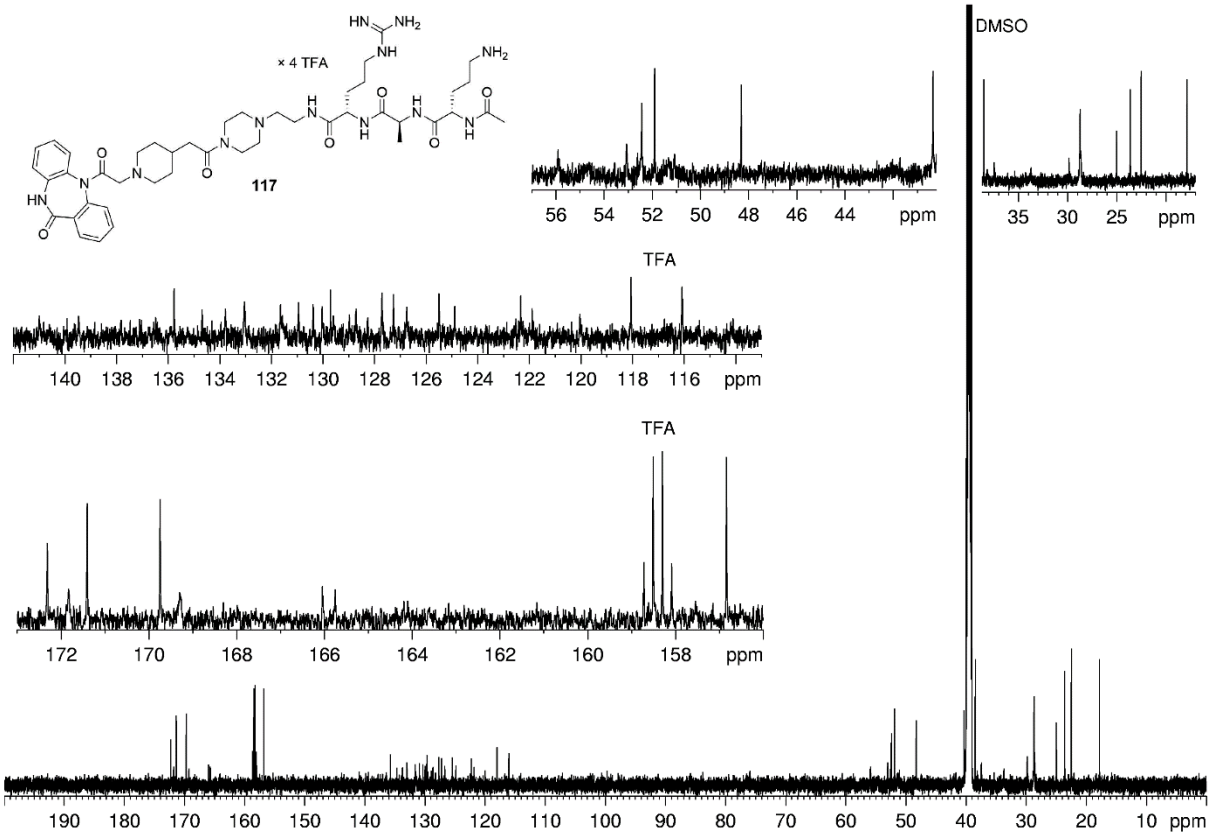
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **116**



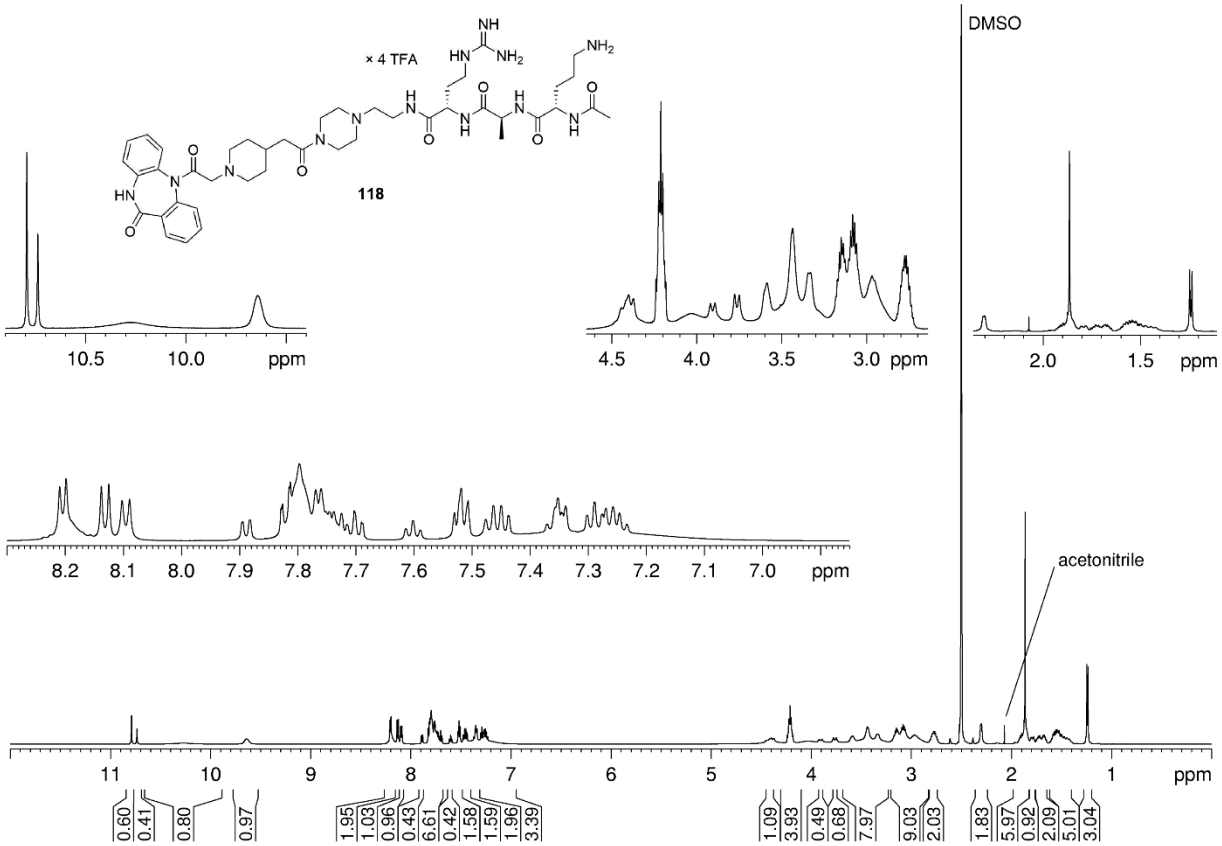
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **117**



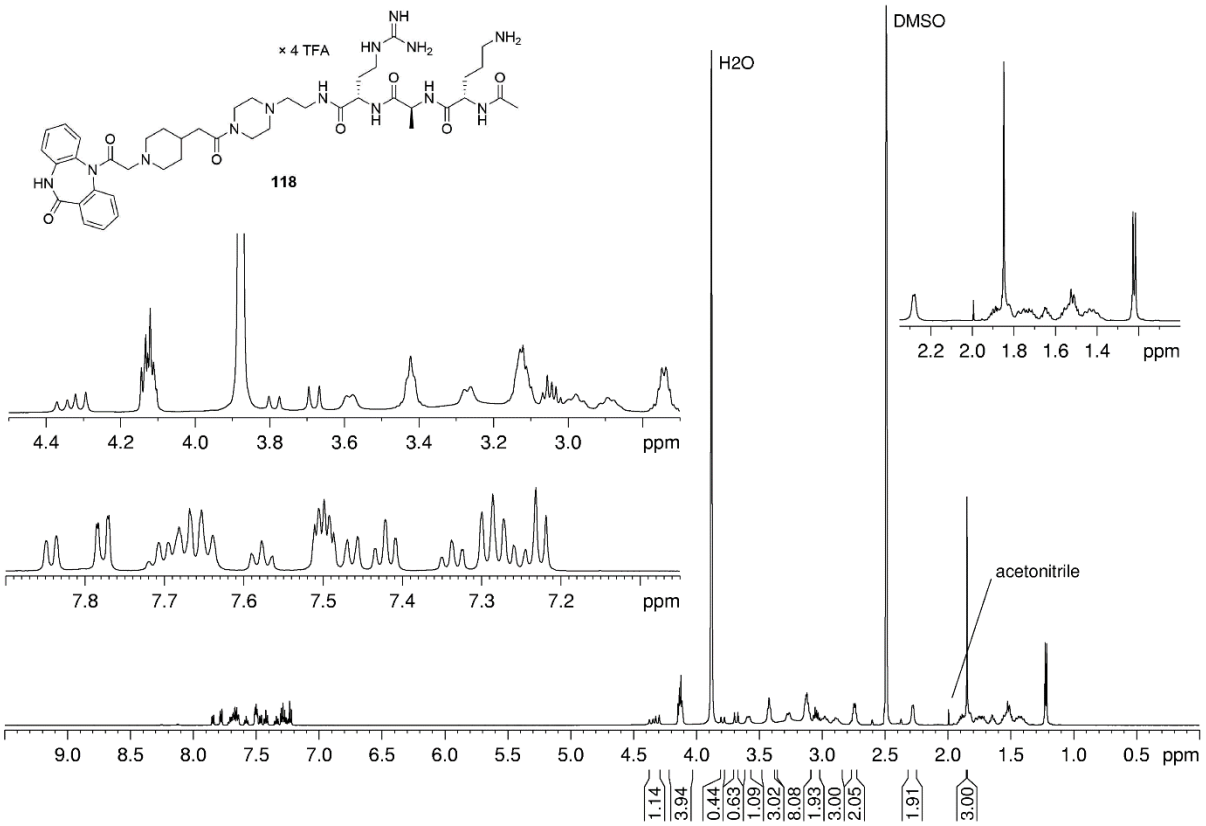
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **117**



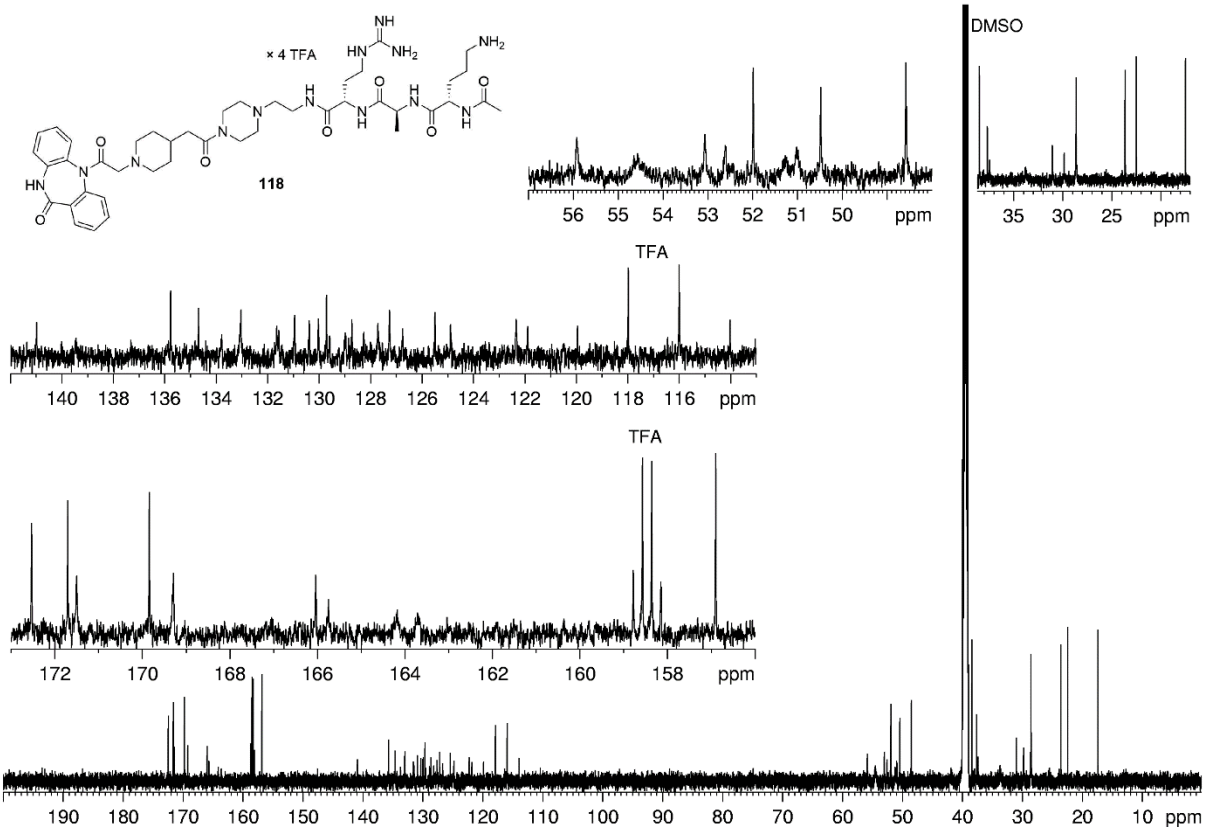
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **117**



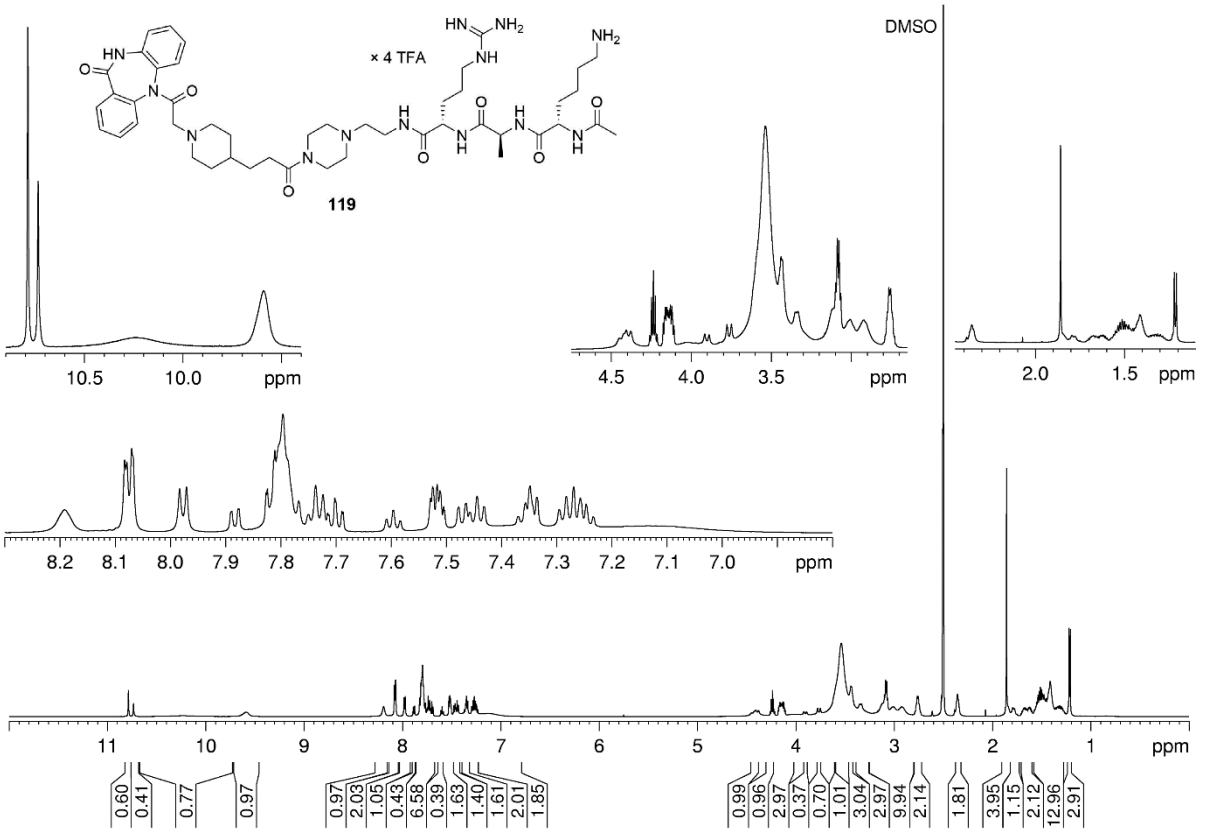
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **118**



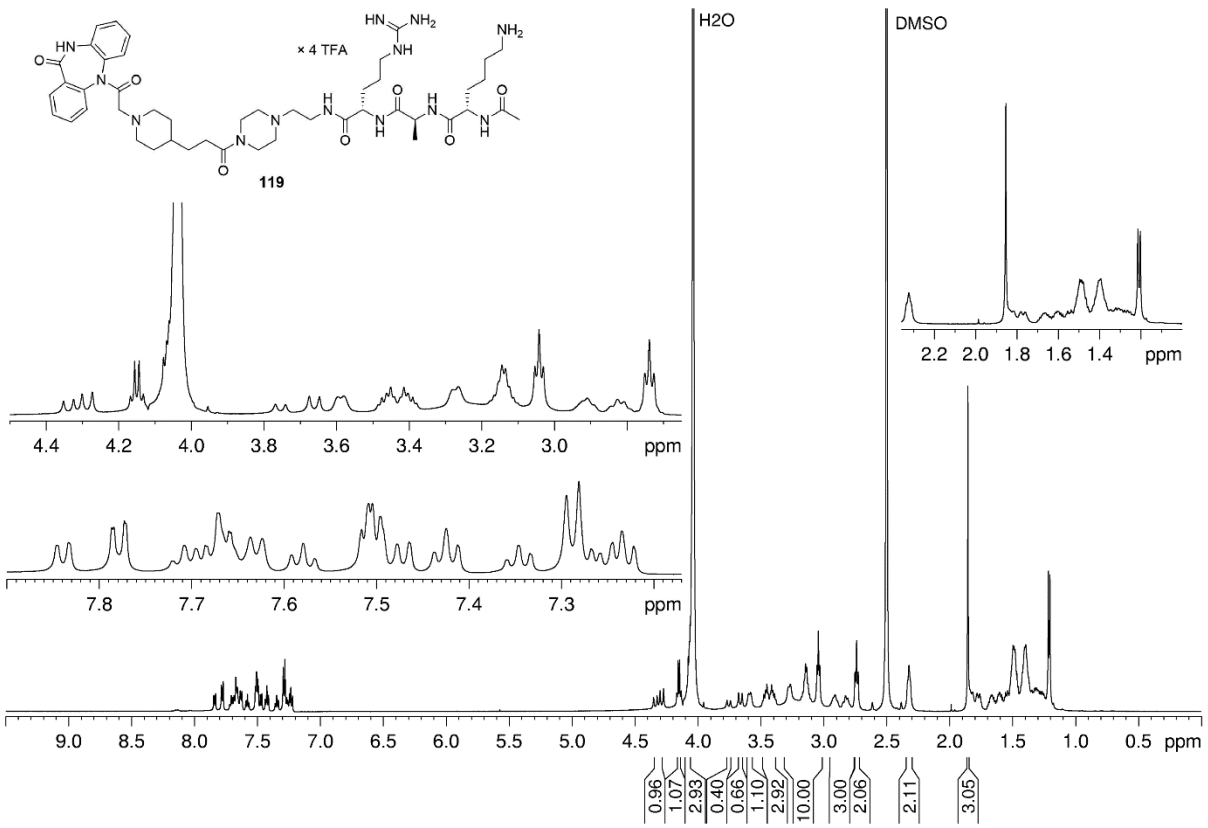
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **118**



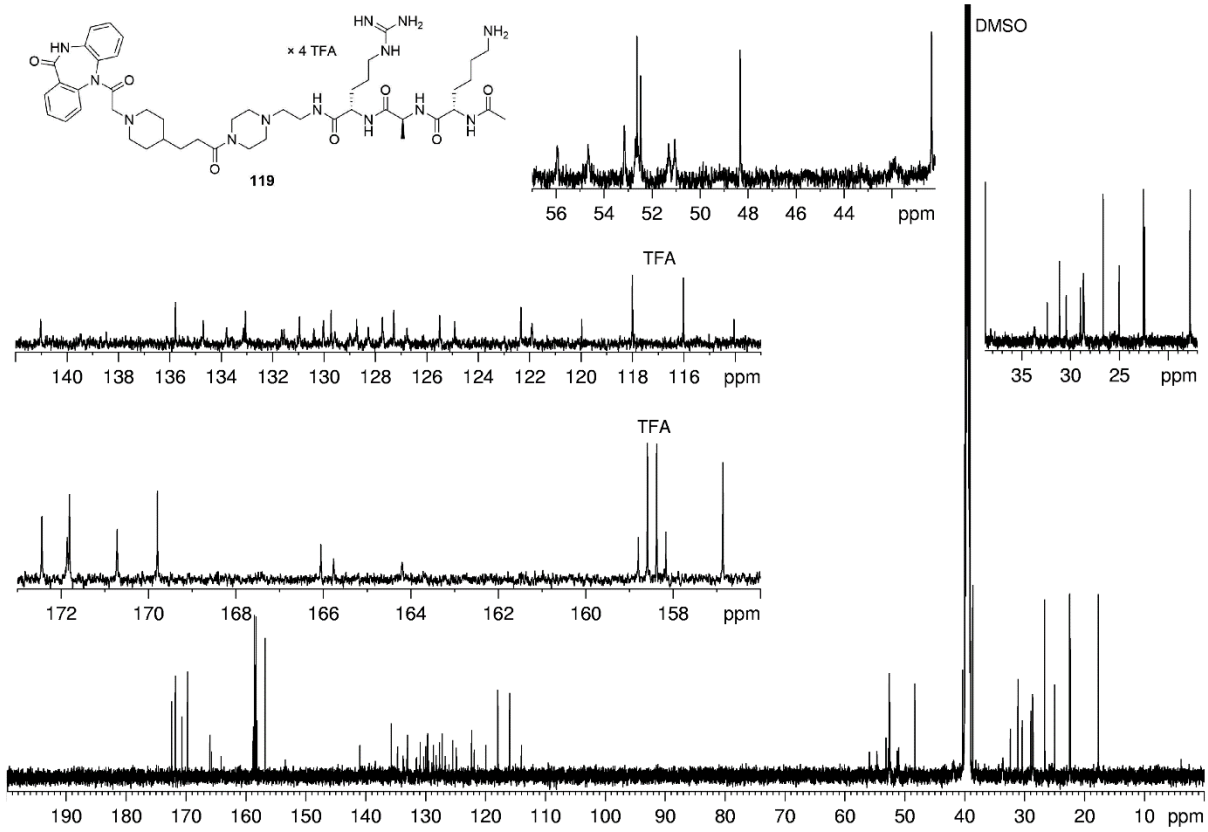
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **118**



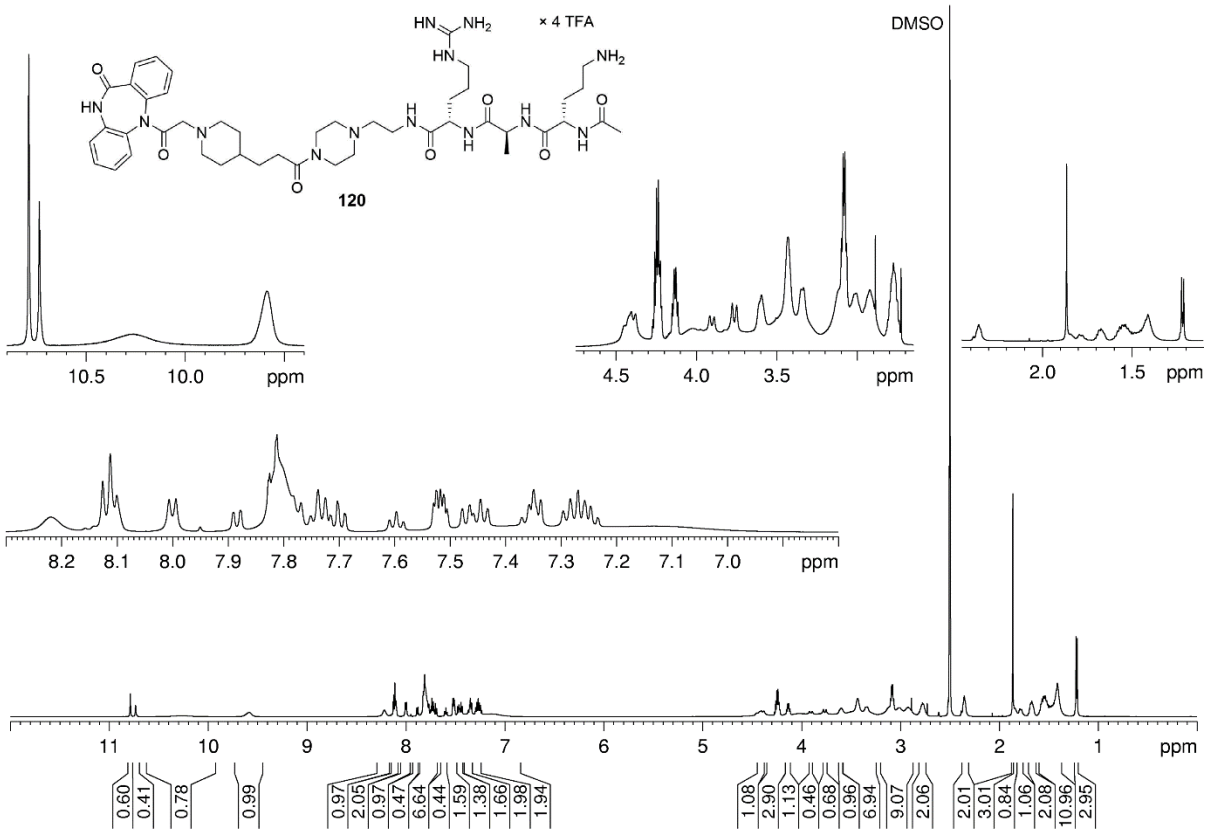
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **119**



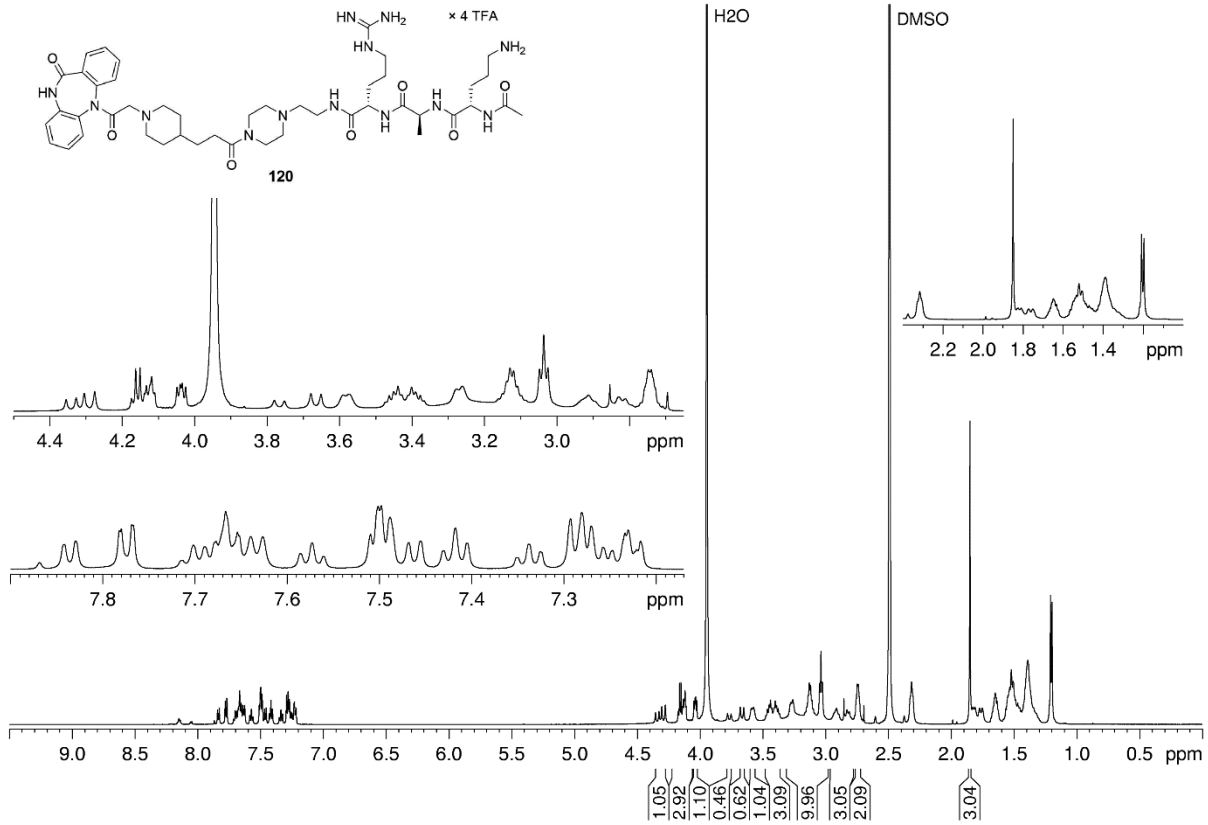
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **119**



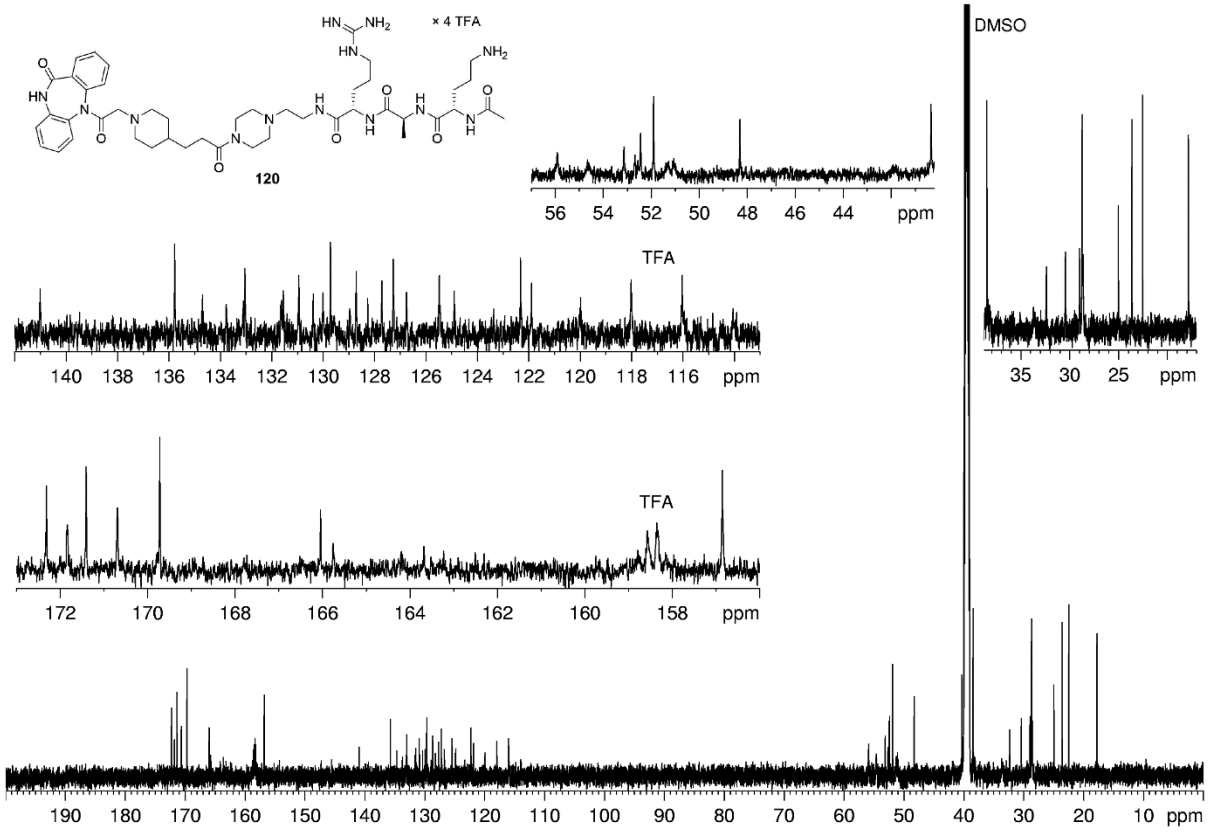
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **119**



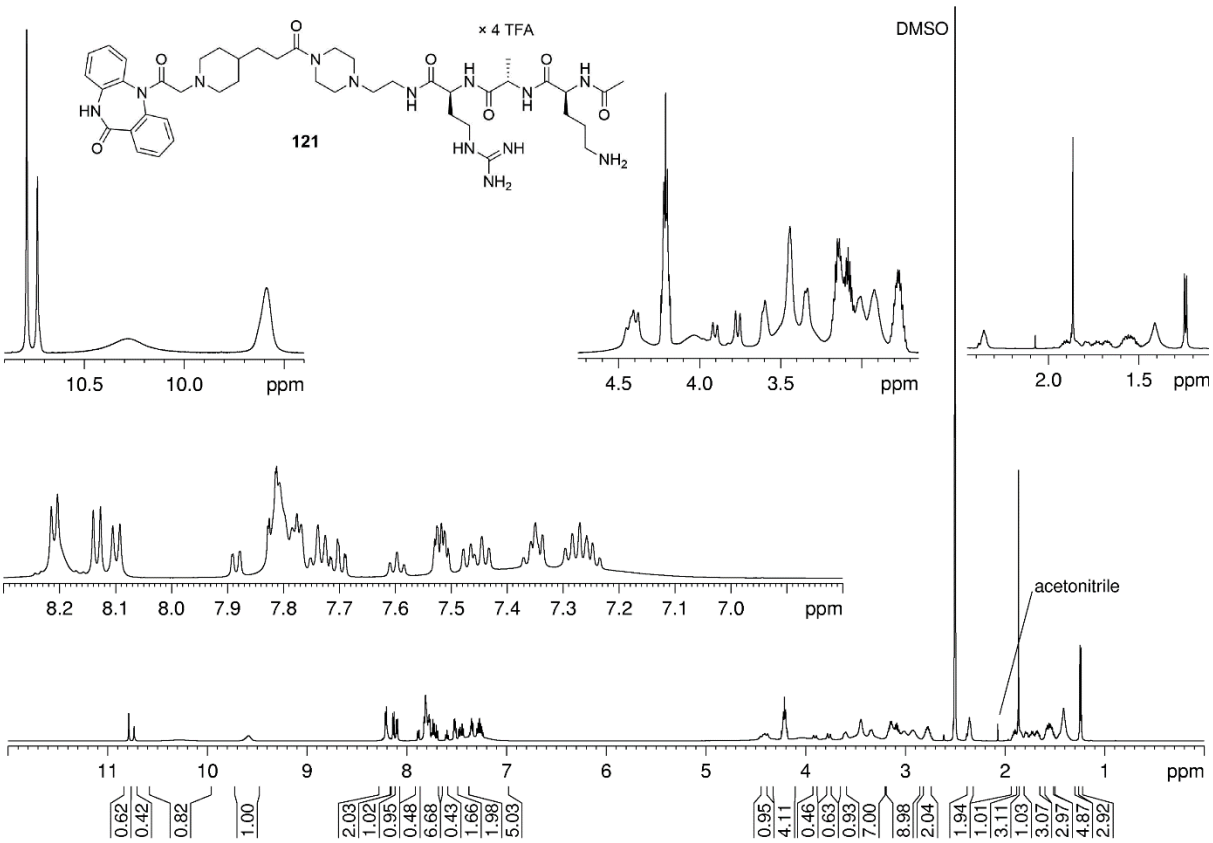
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **120**



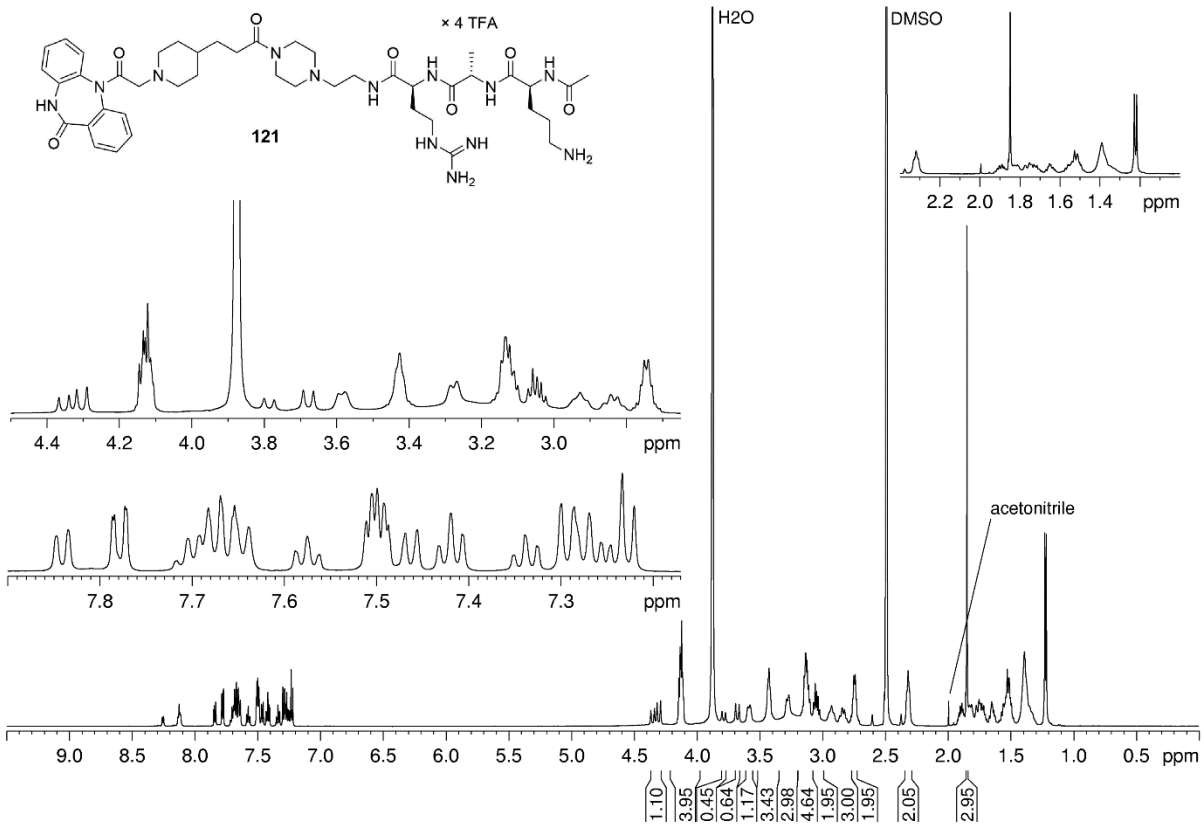
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **120**



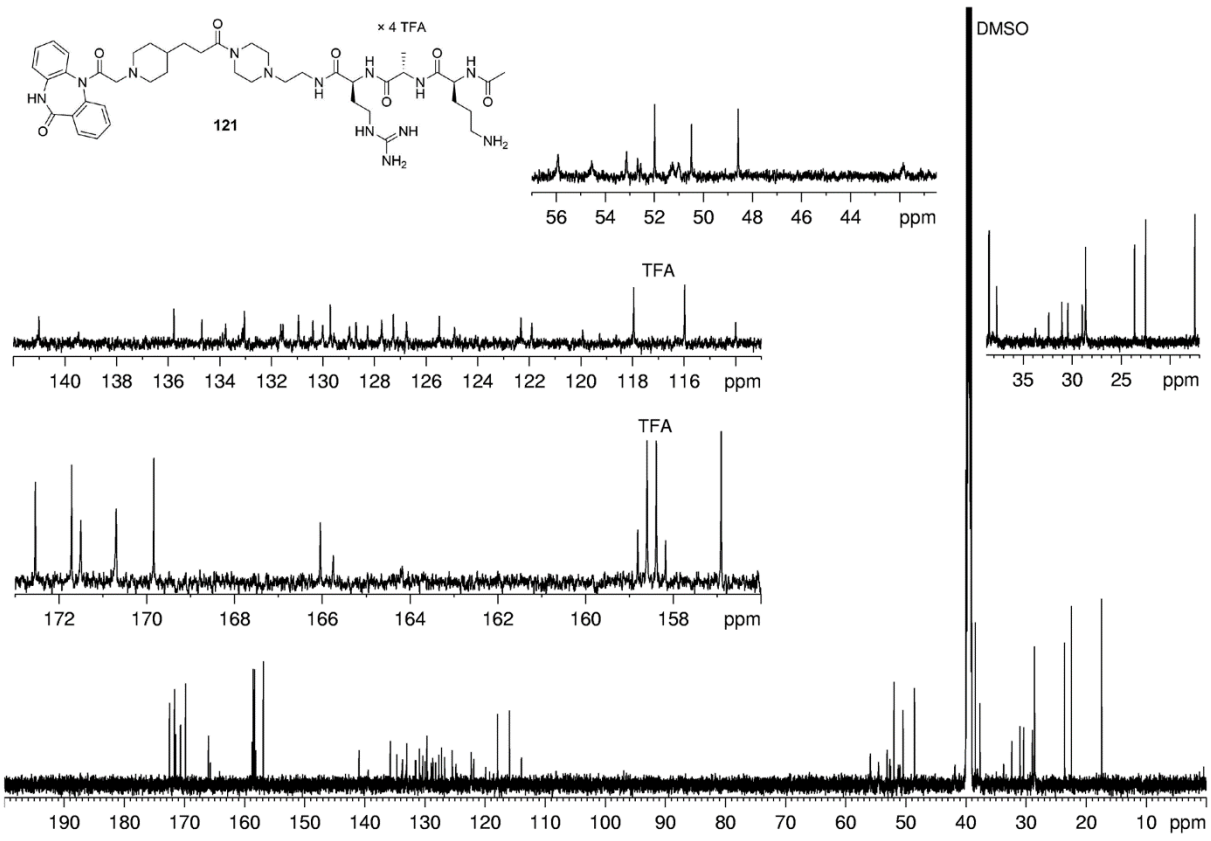
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **120**



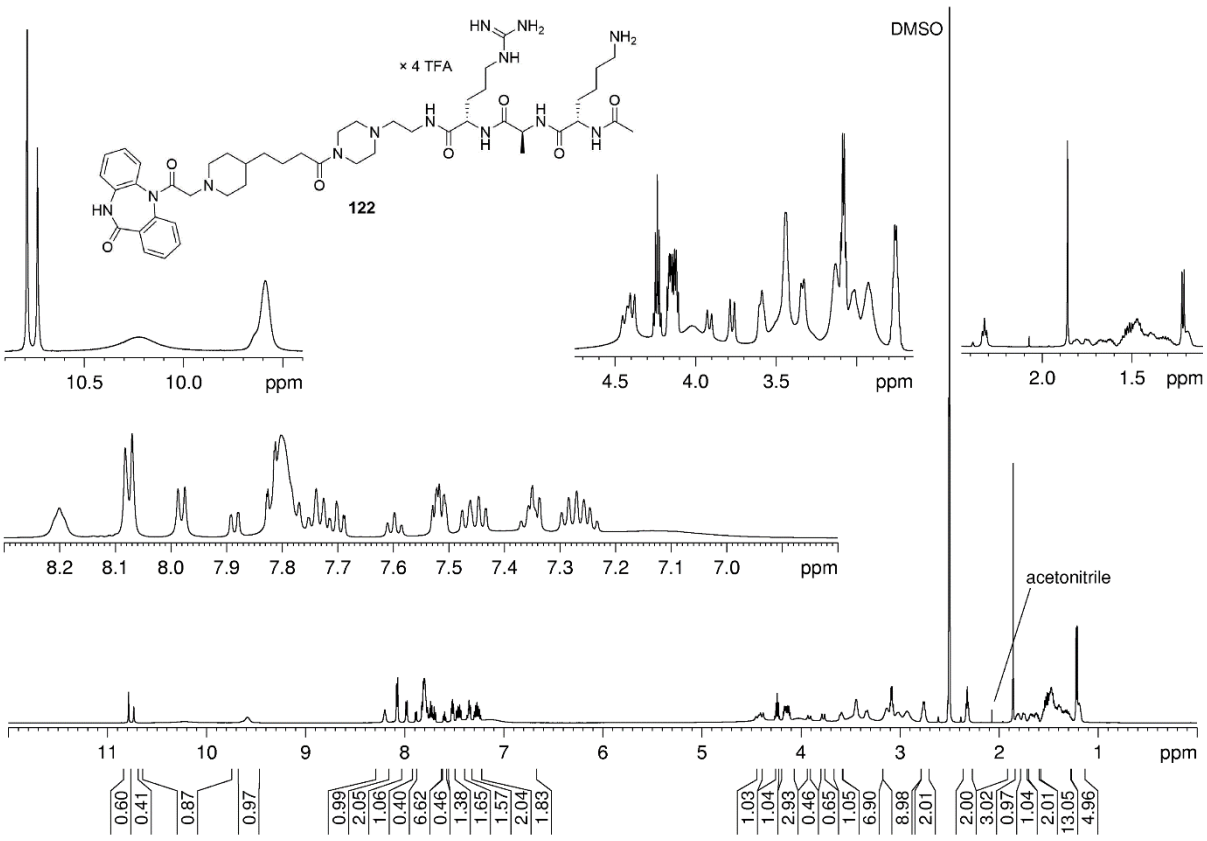
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **121**



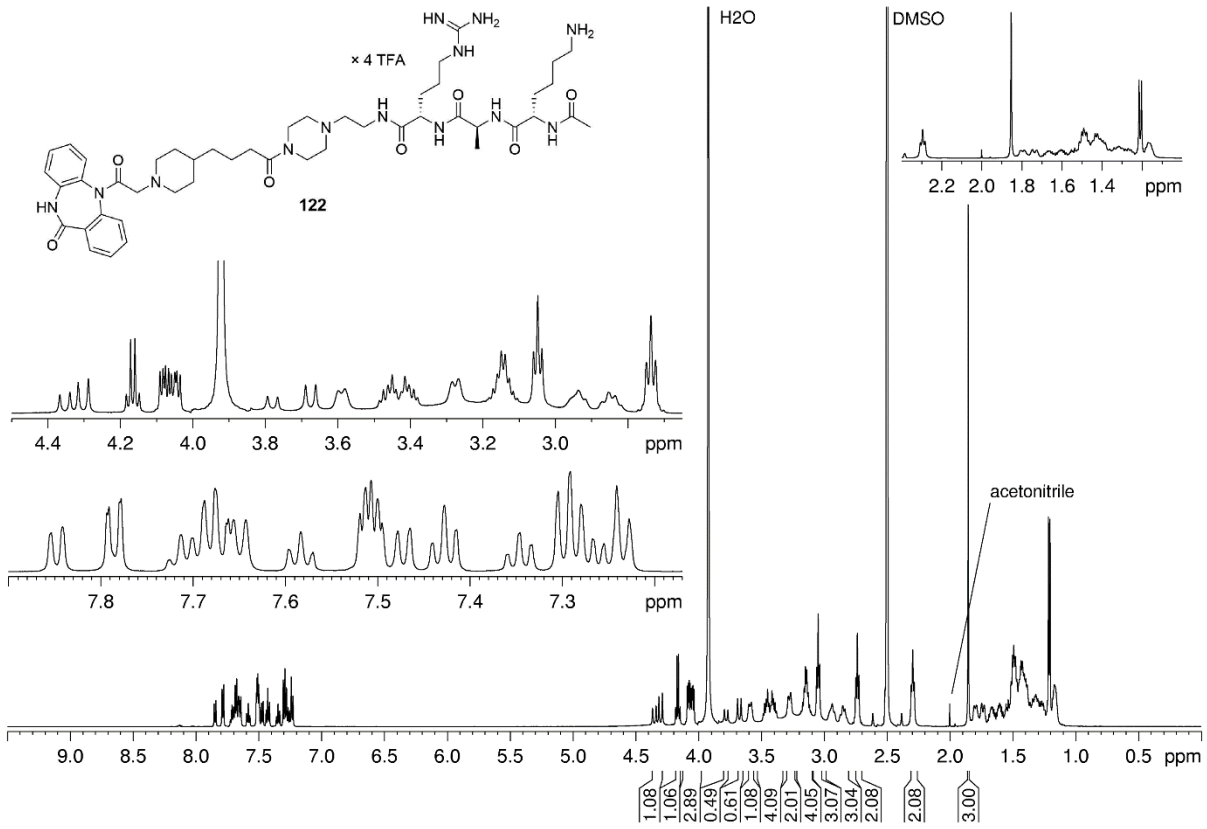
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **121**



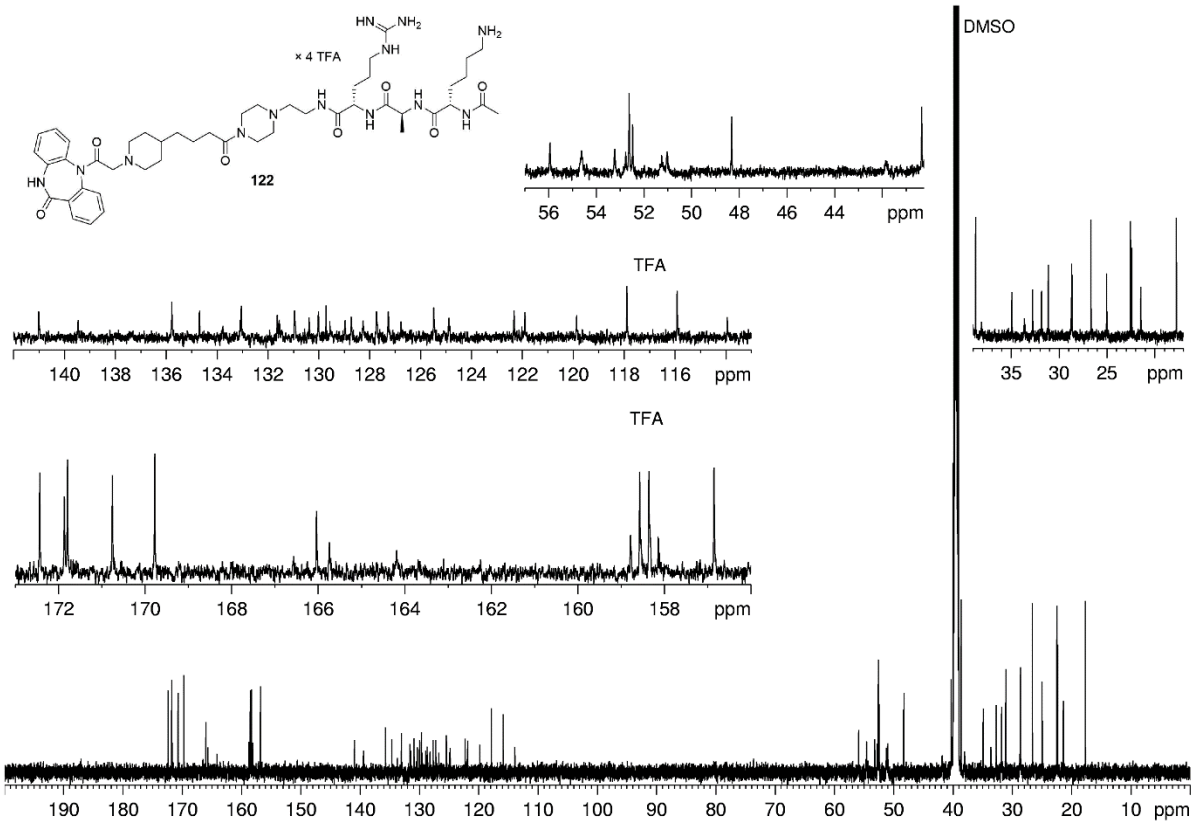
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **121**



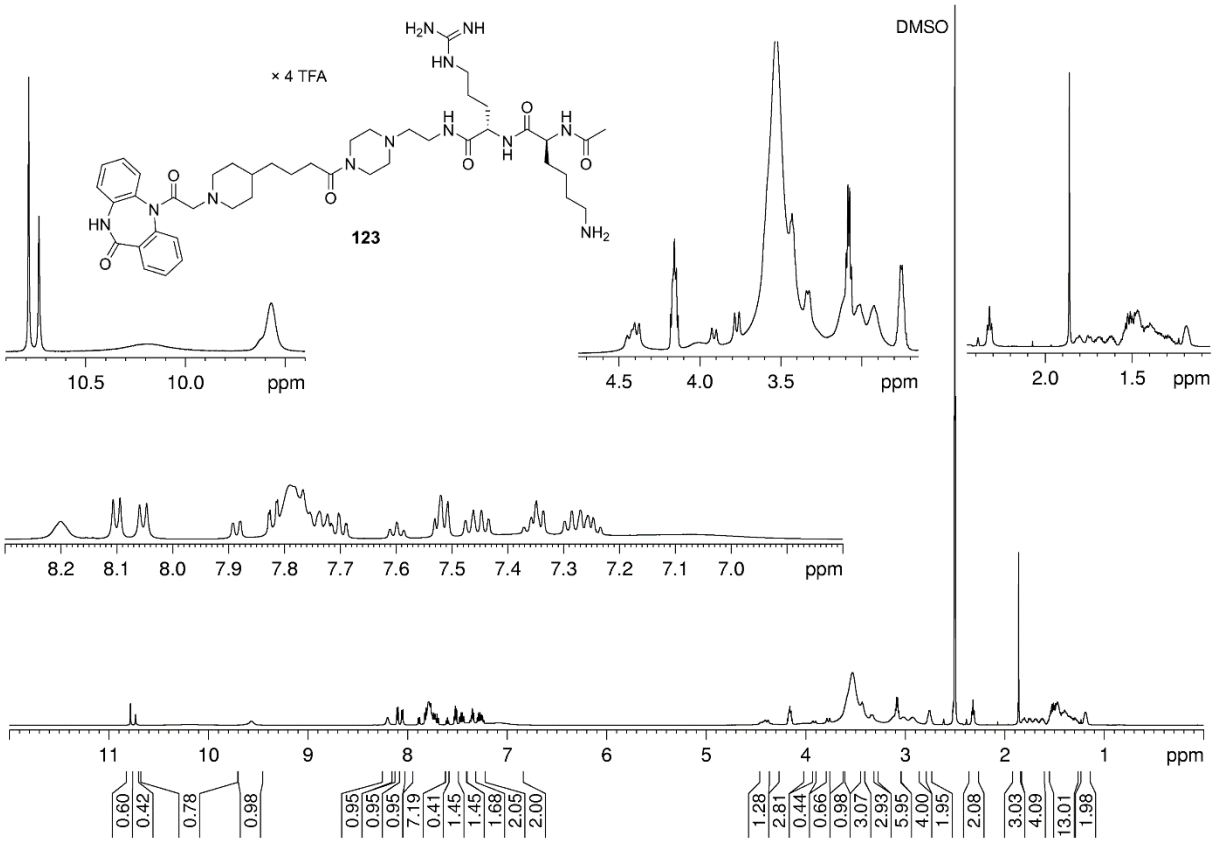
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **122**



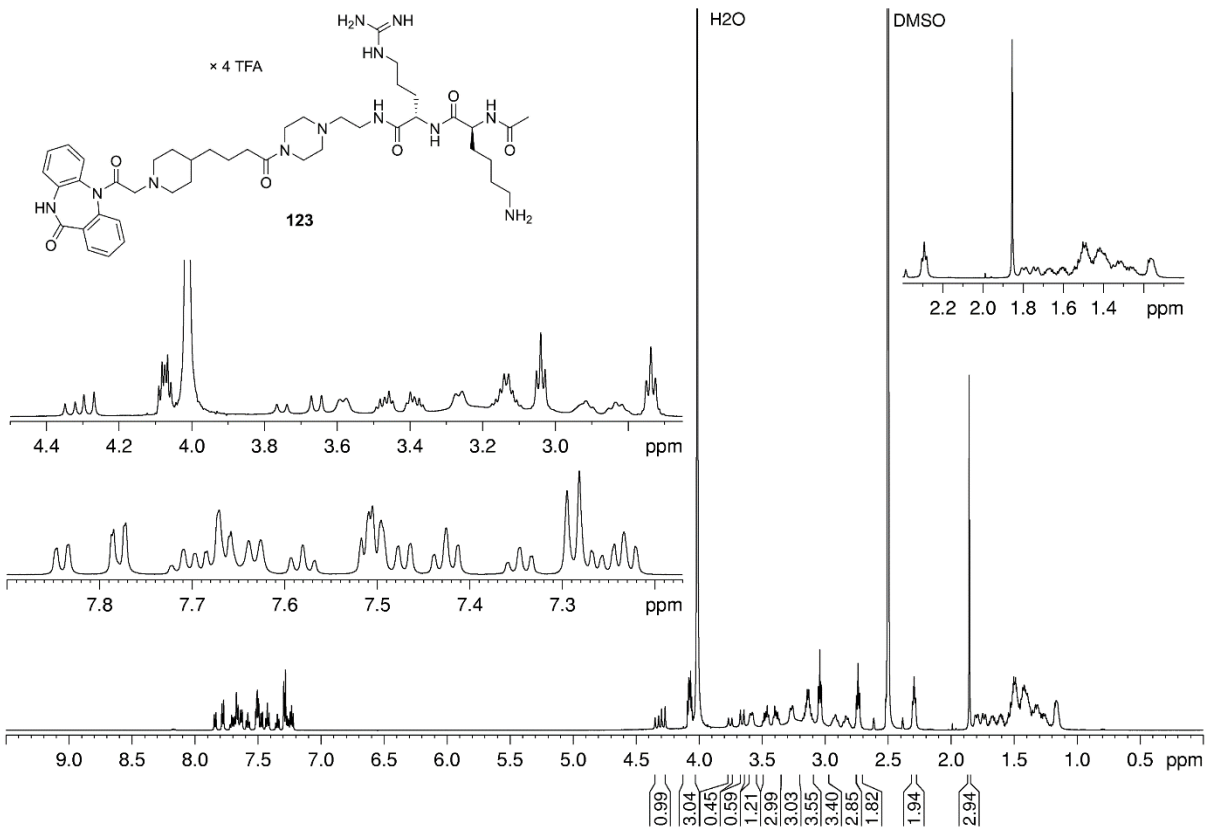
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **122**



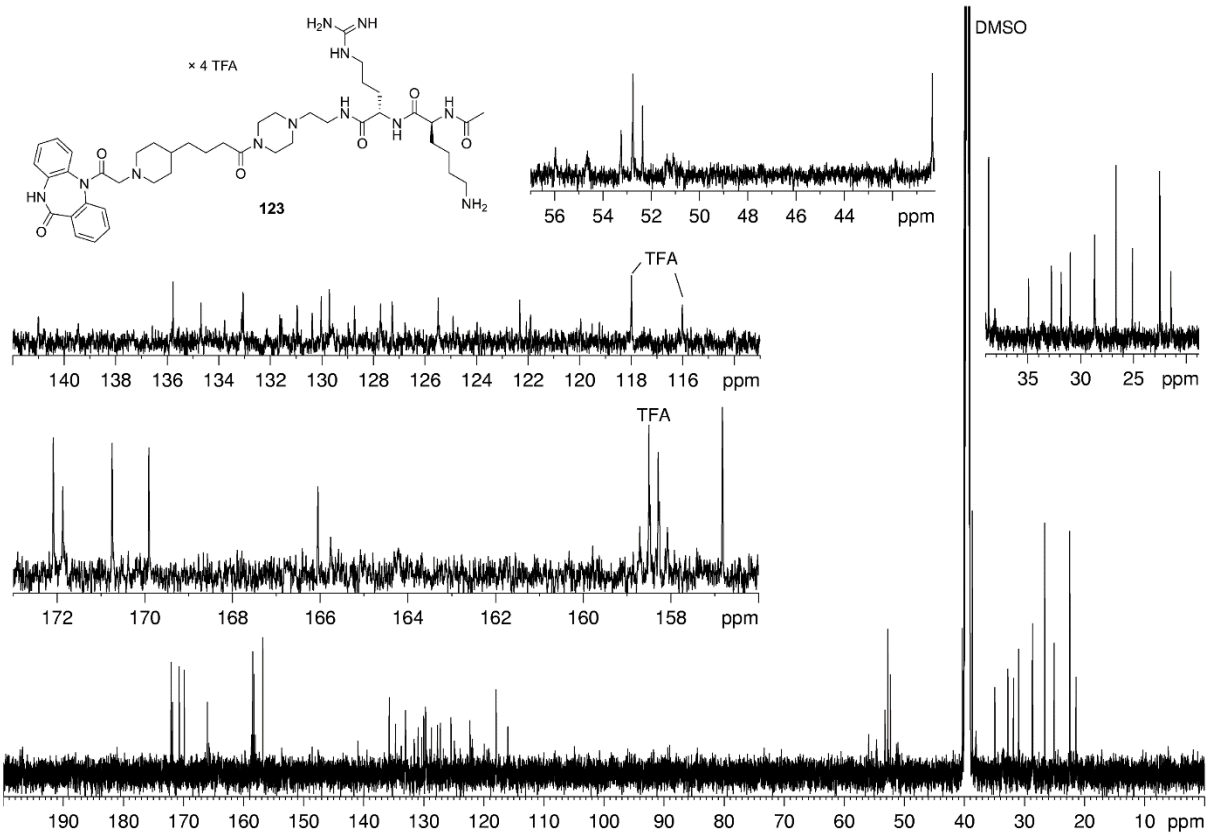
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **122**



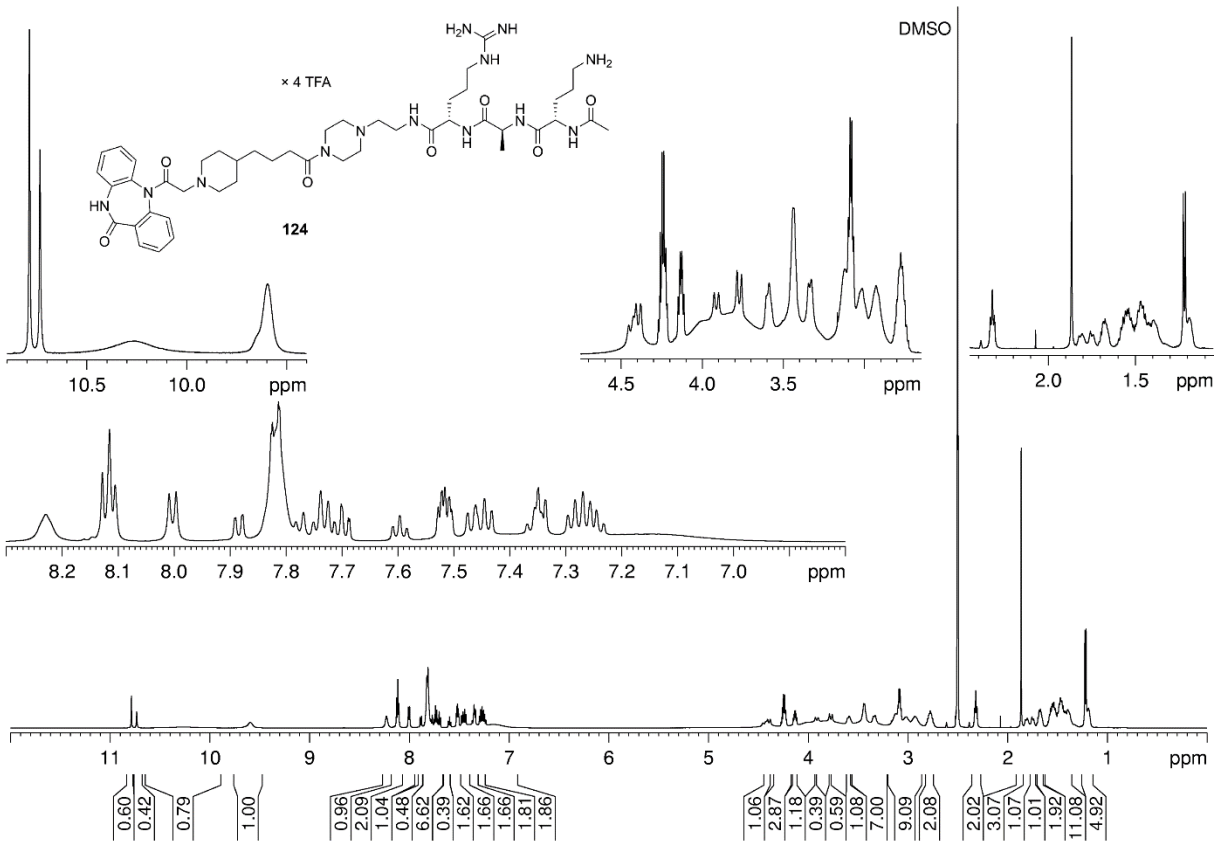
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **123**



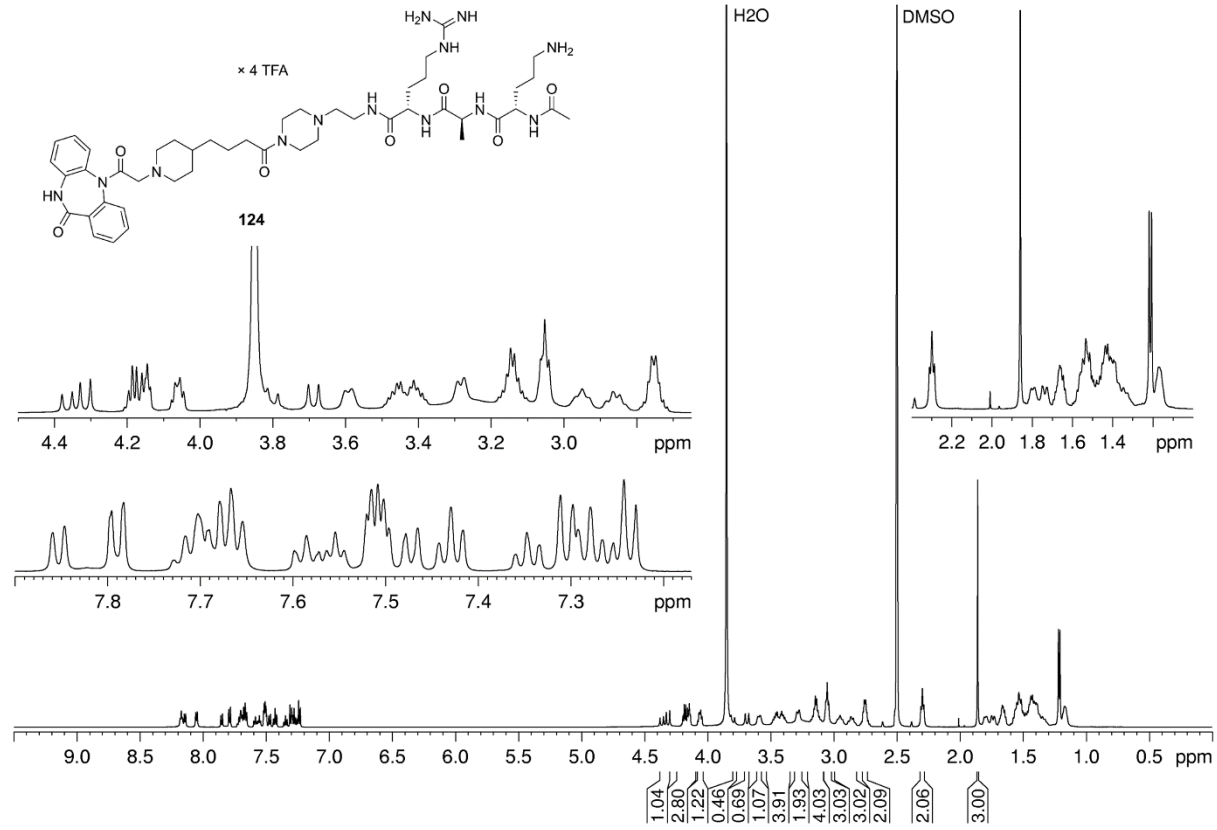
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **123**



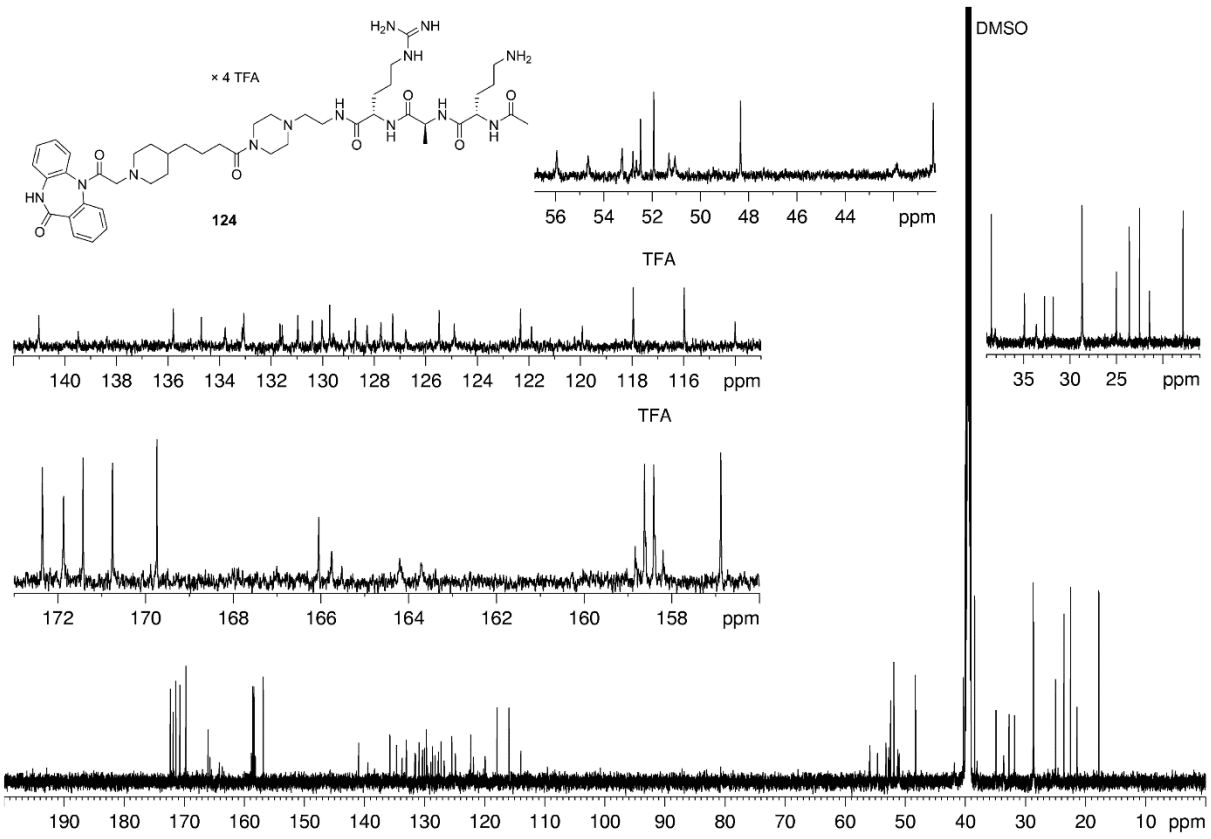
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **123**



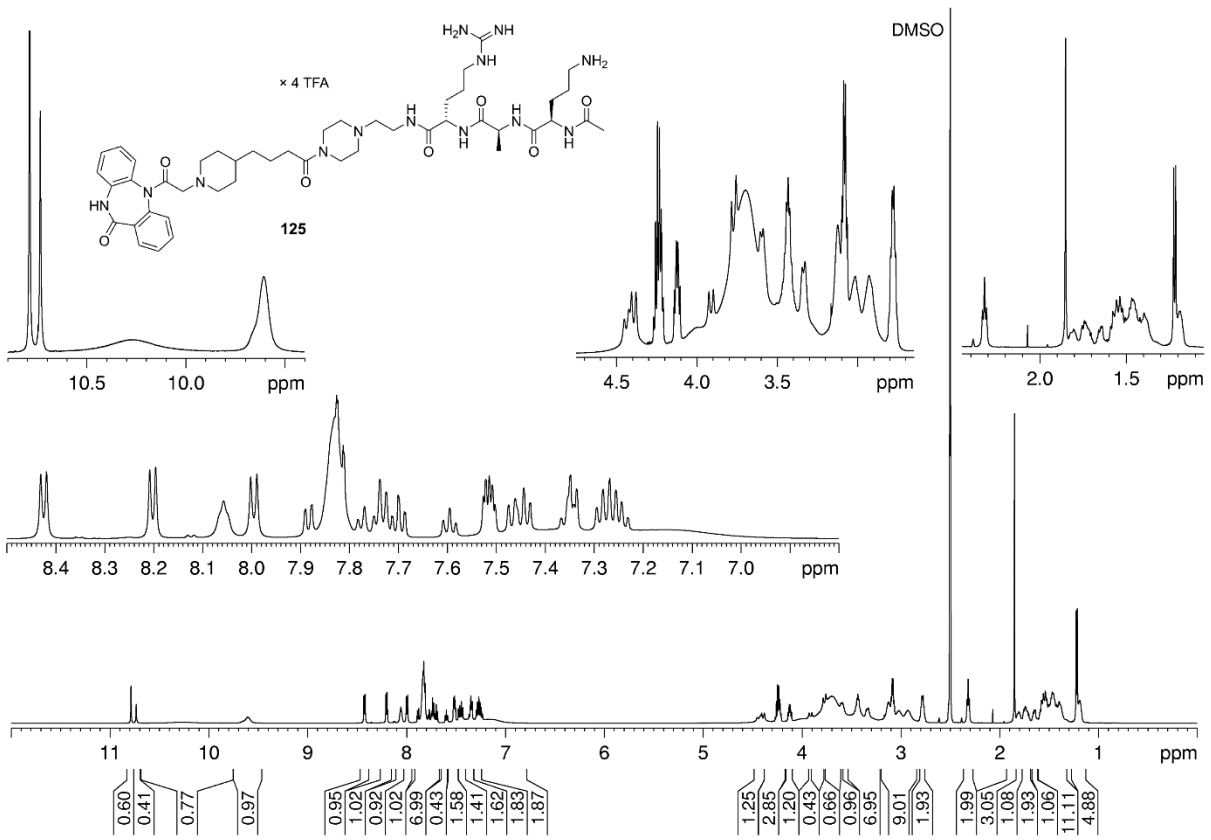
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **124**



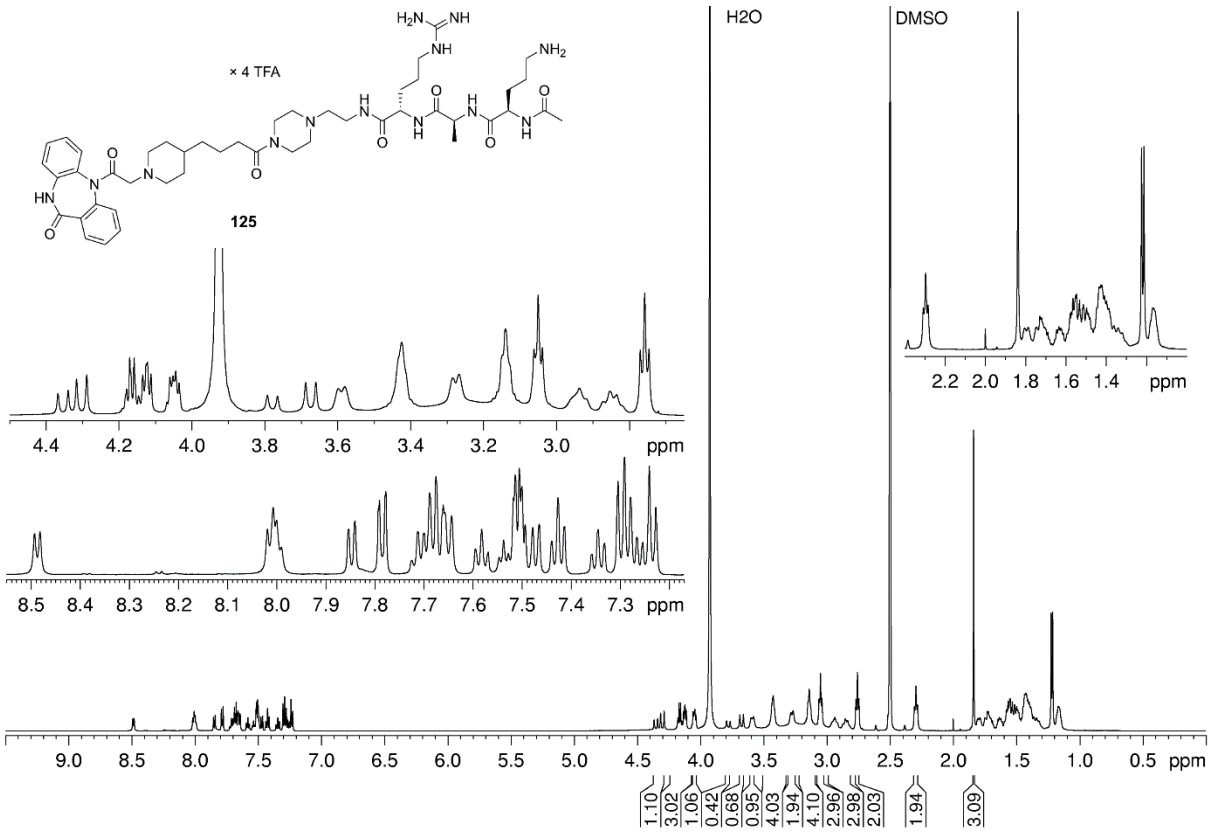
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **124**



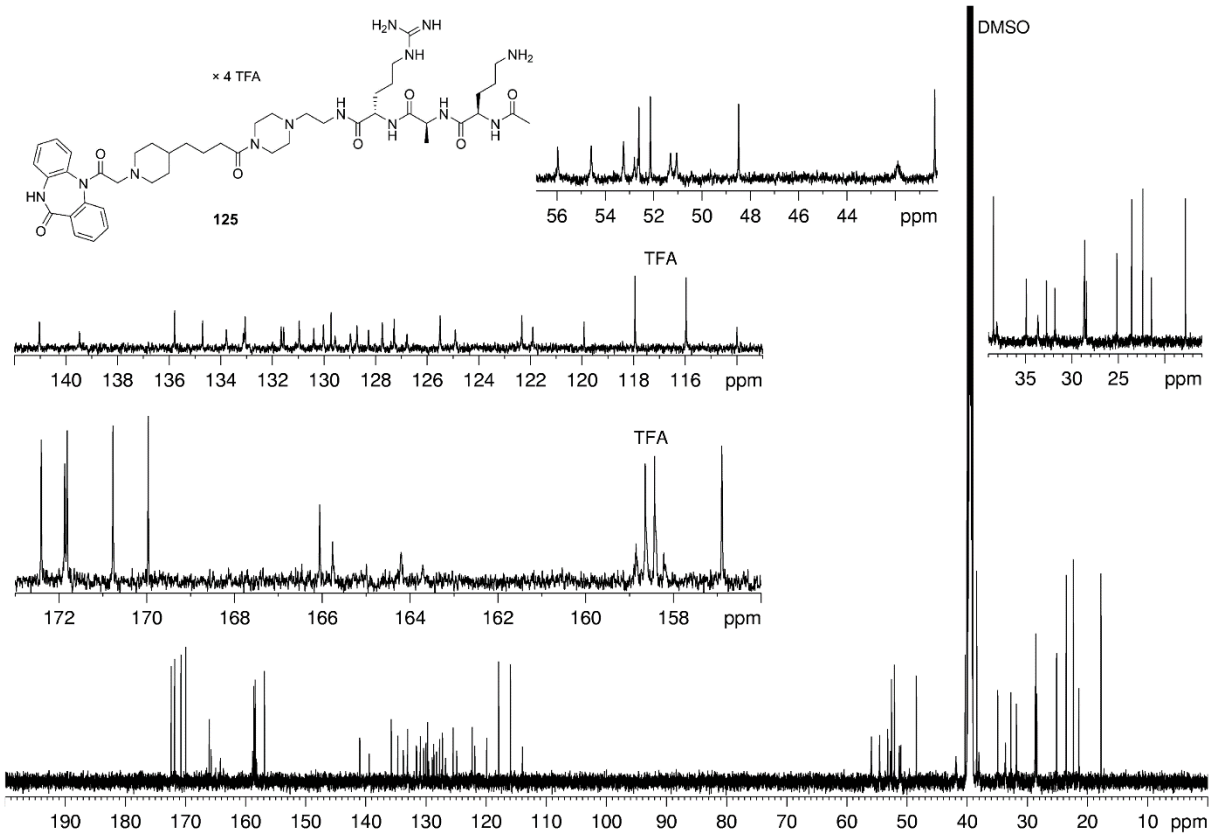
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **124**



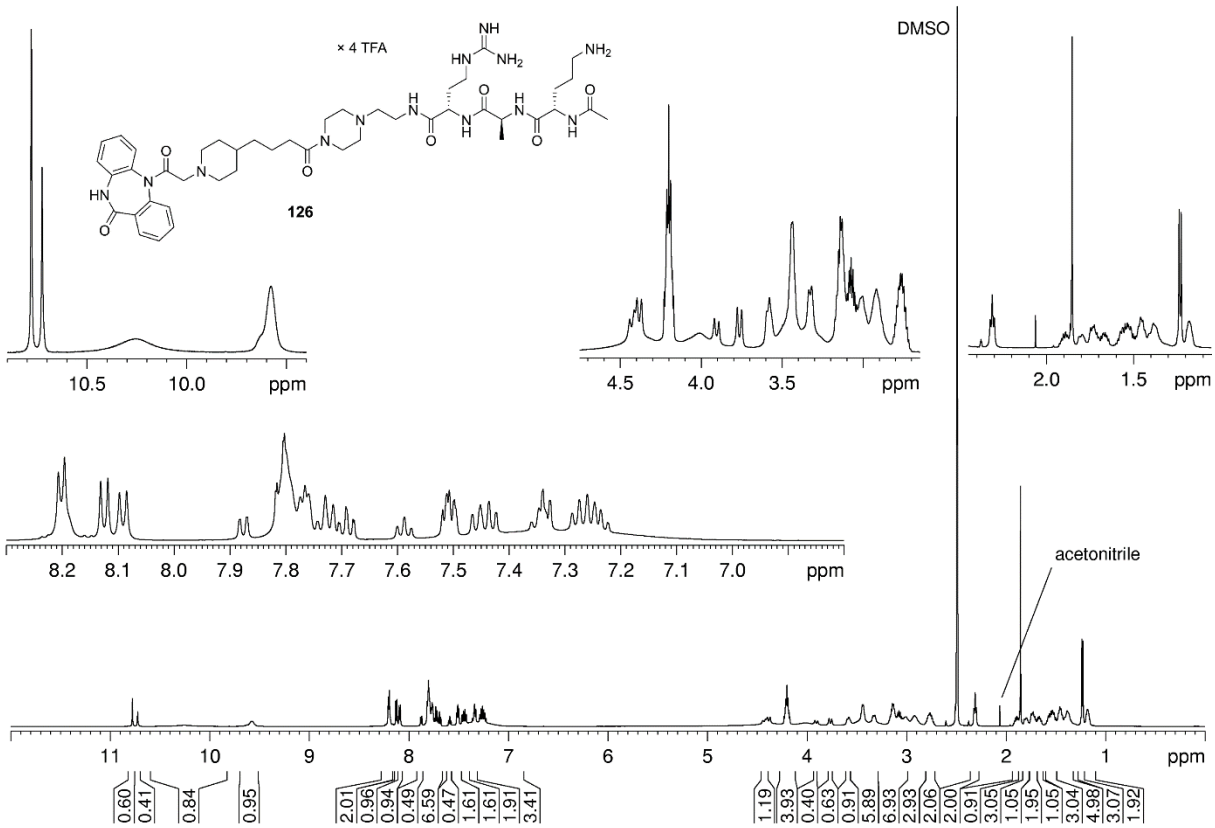
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **125**



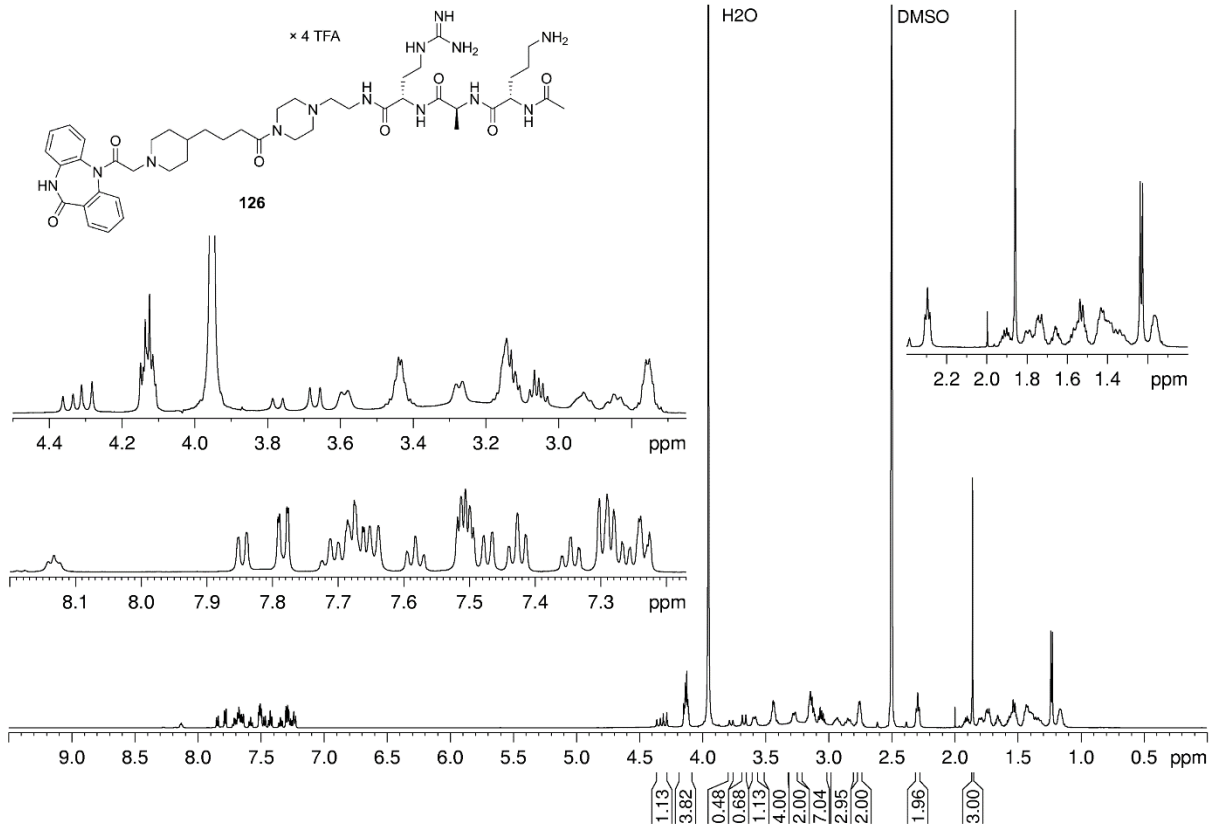
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **125**



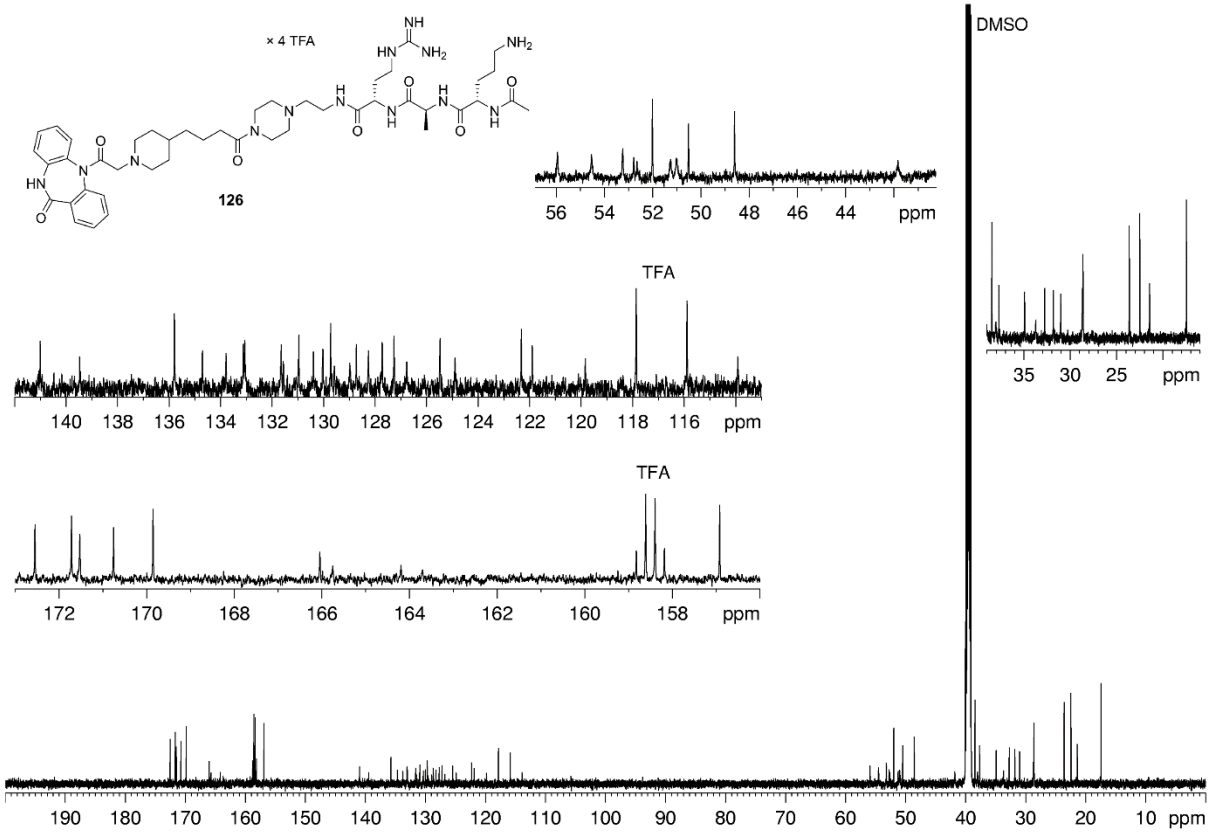
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **125**



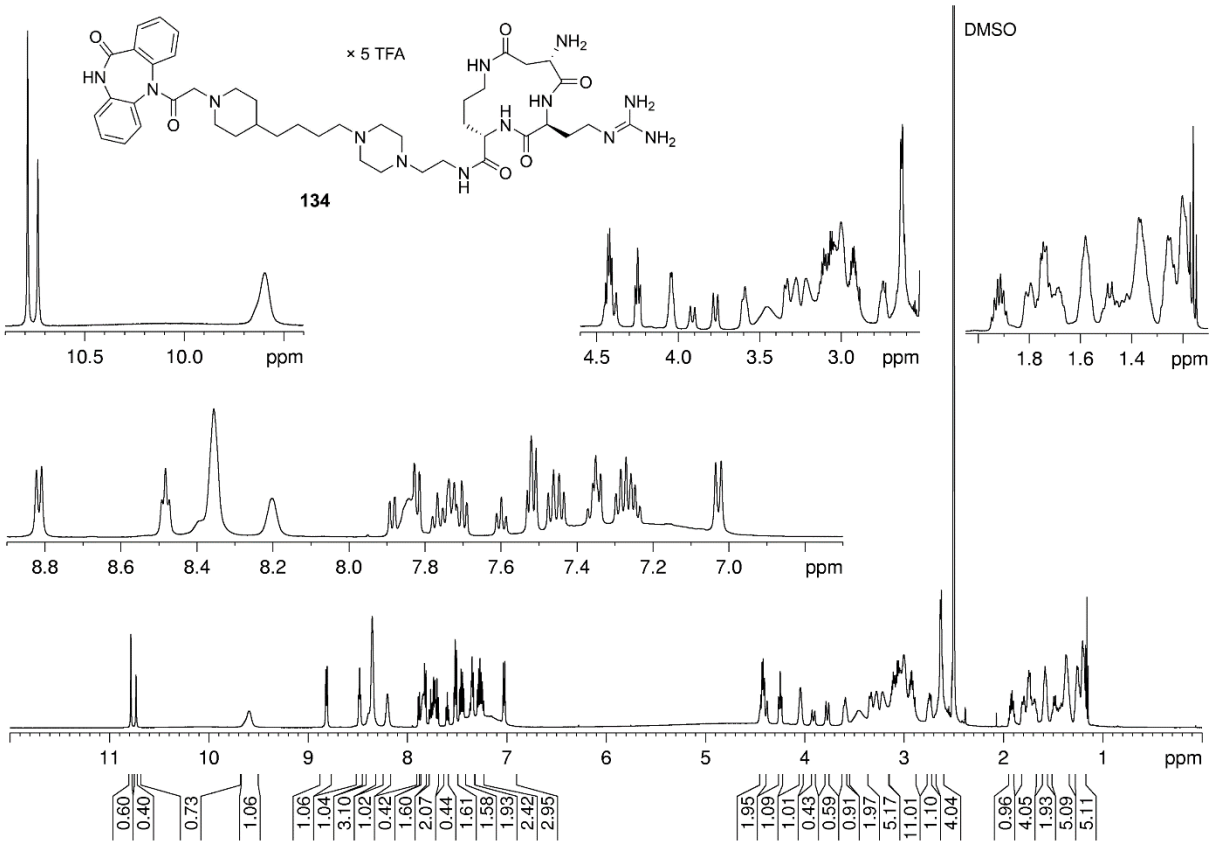
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **126**



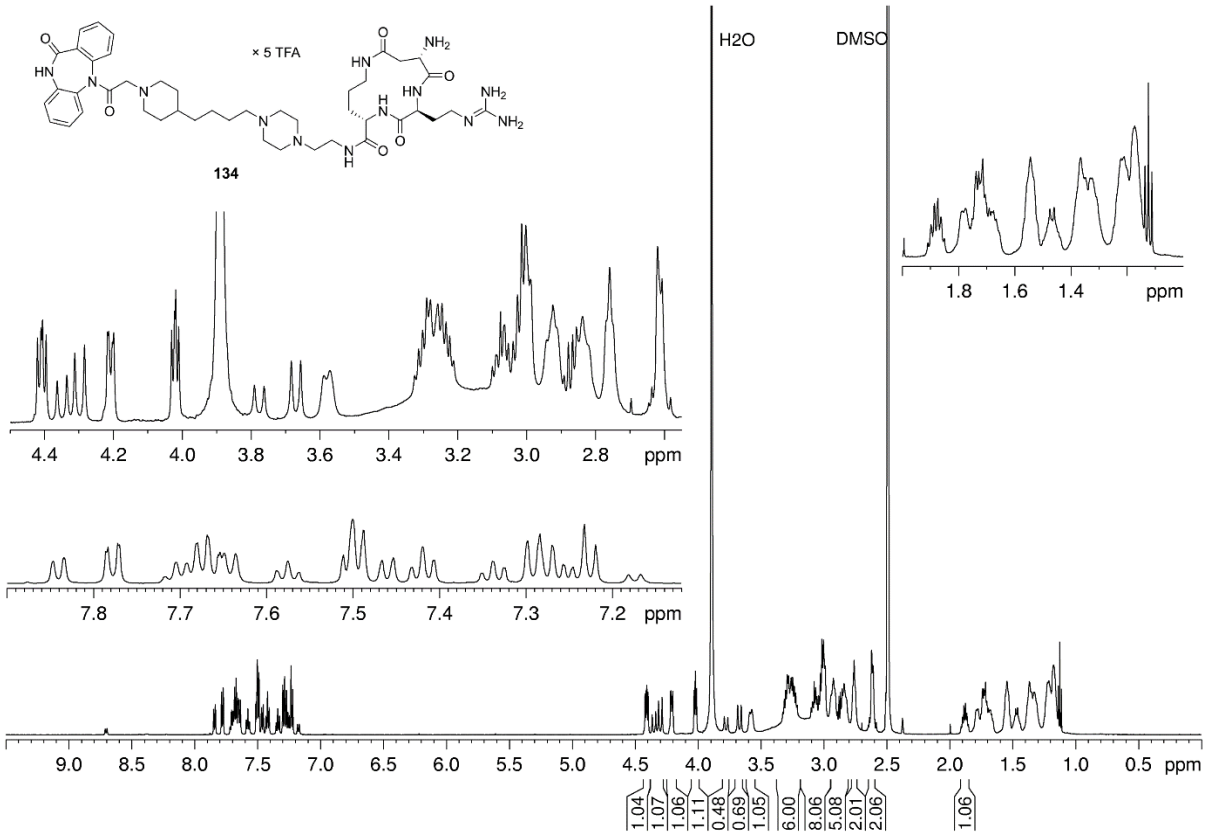
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **126**



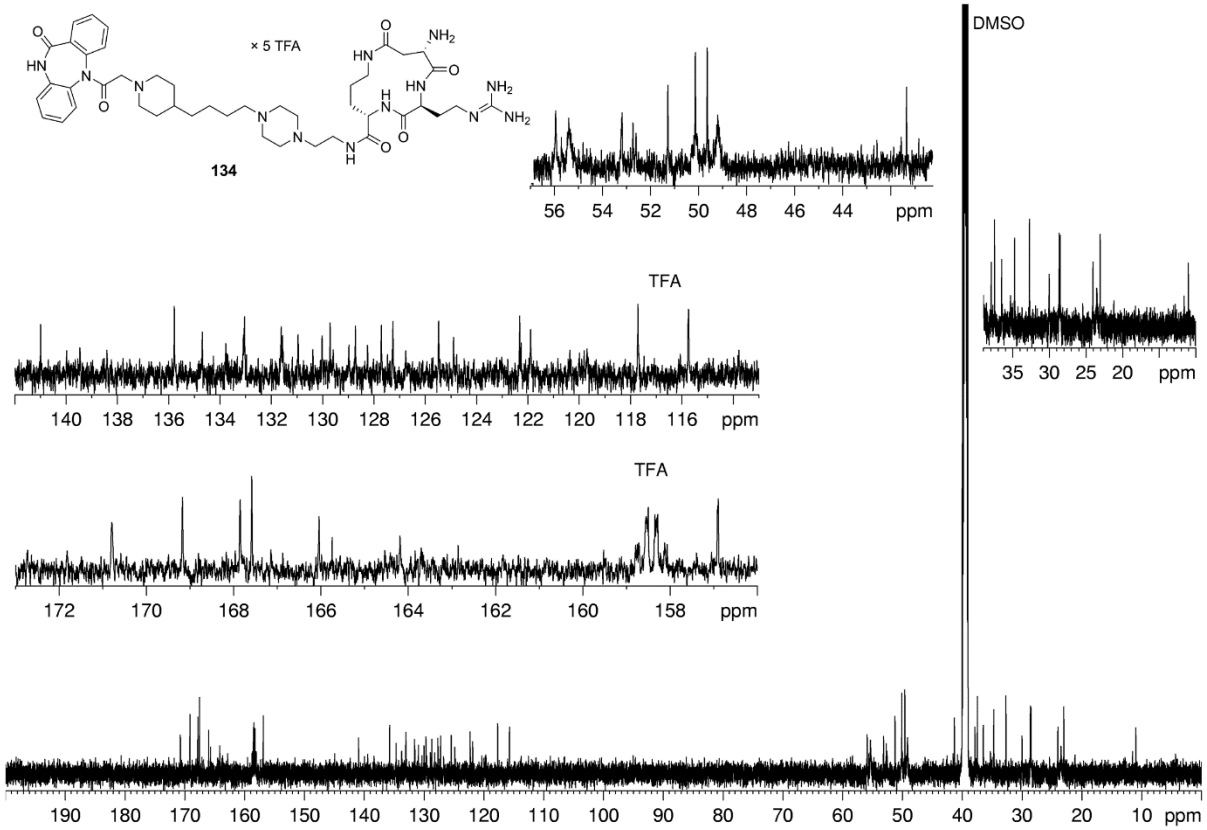
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **126**



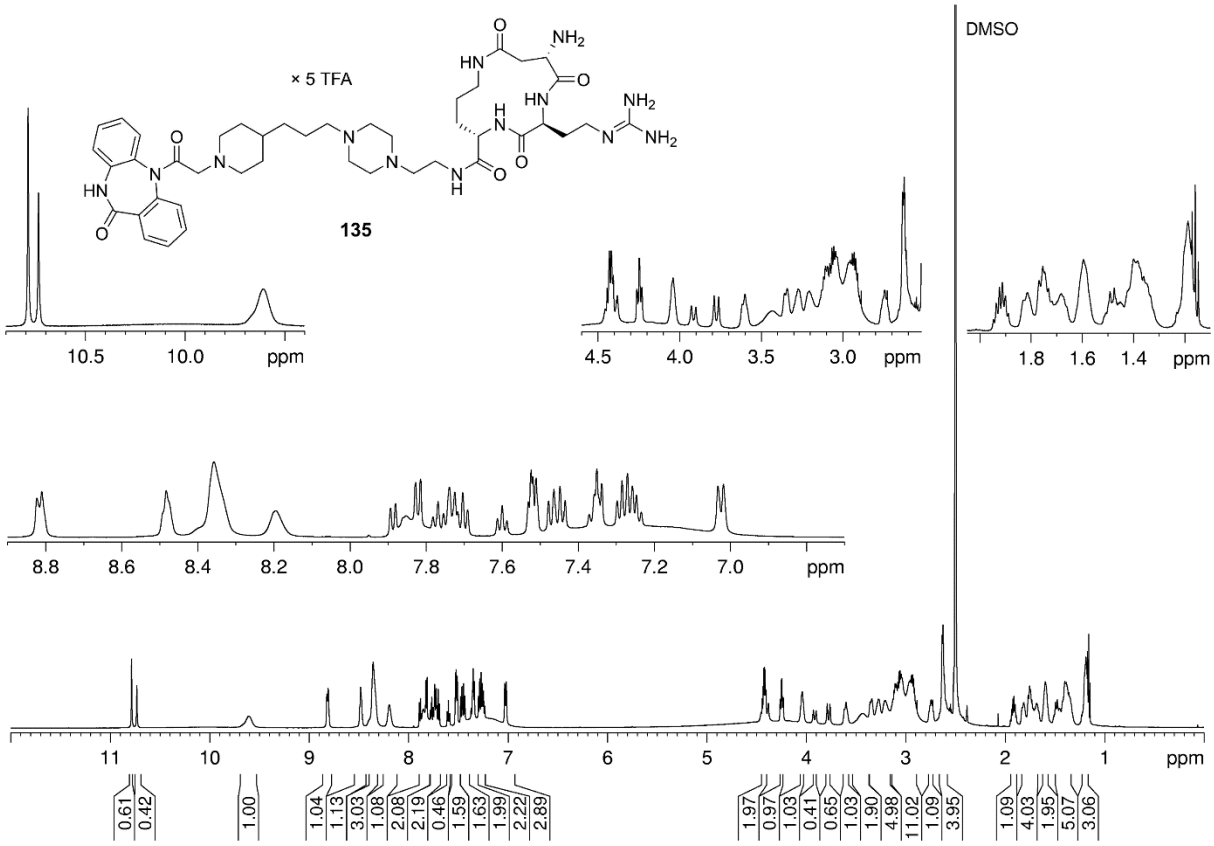
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **134**



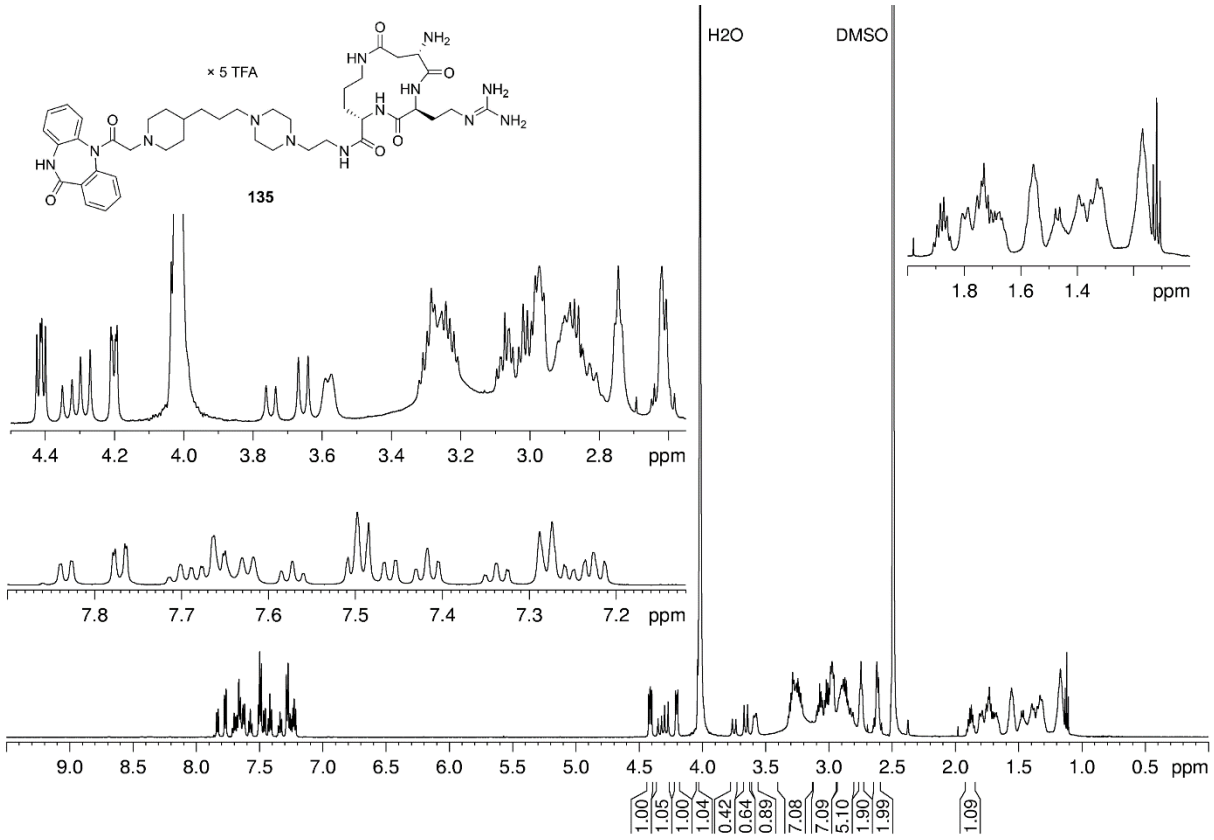
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **134**



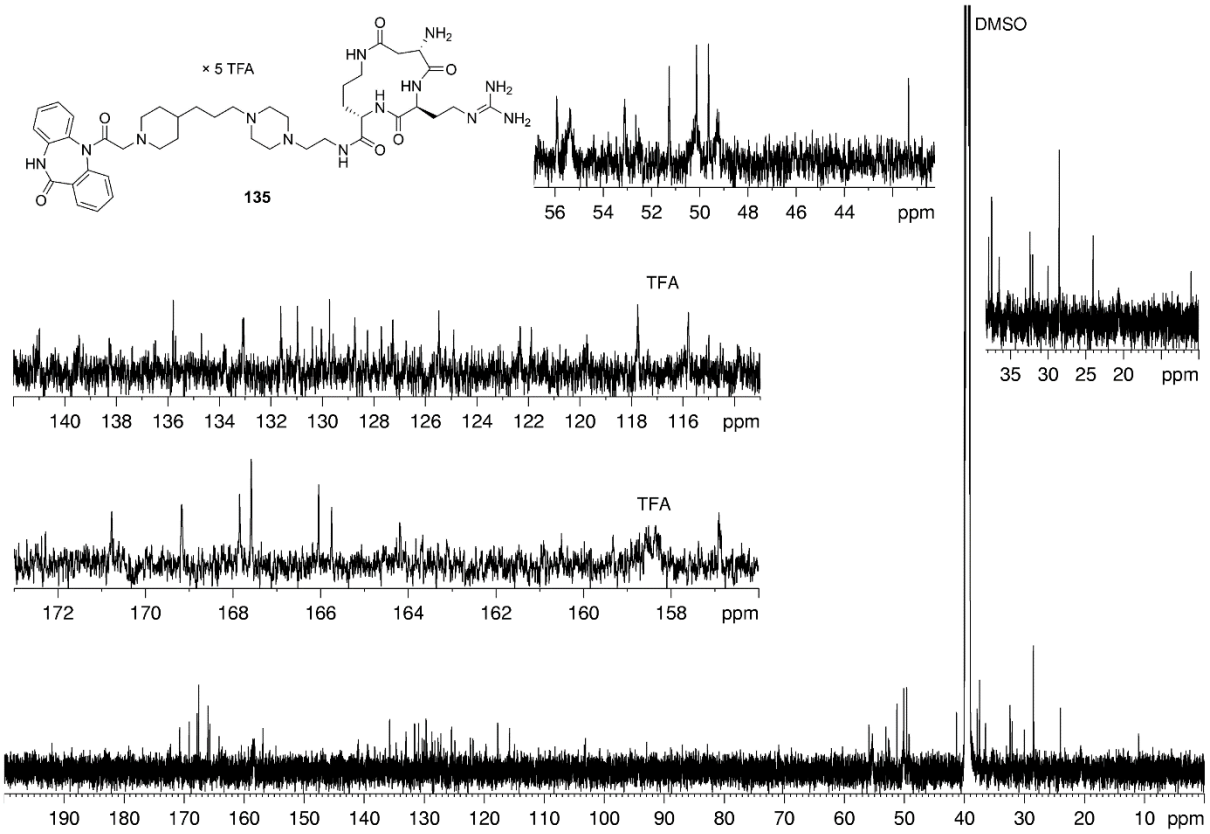
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **134**



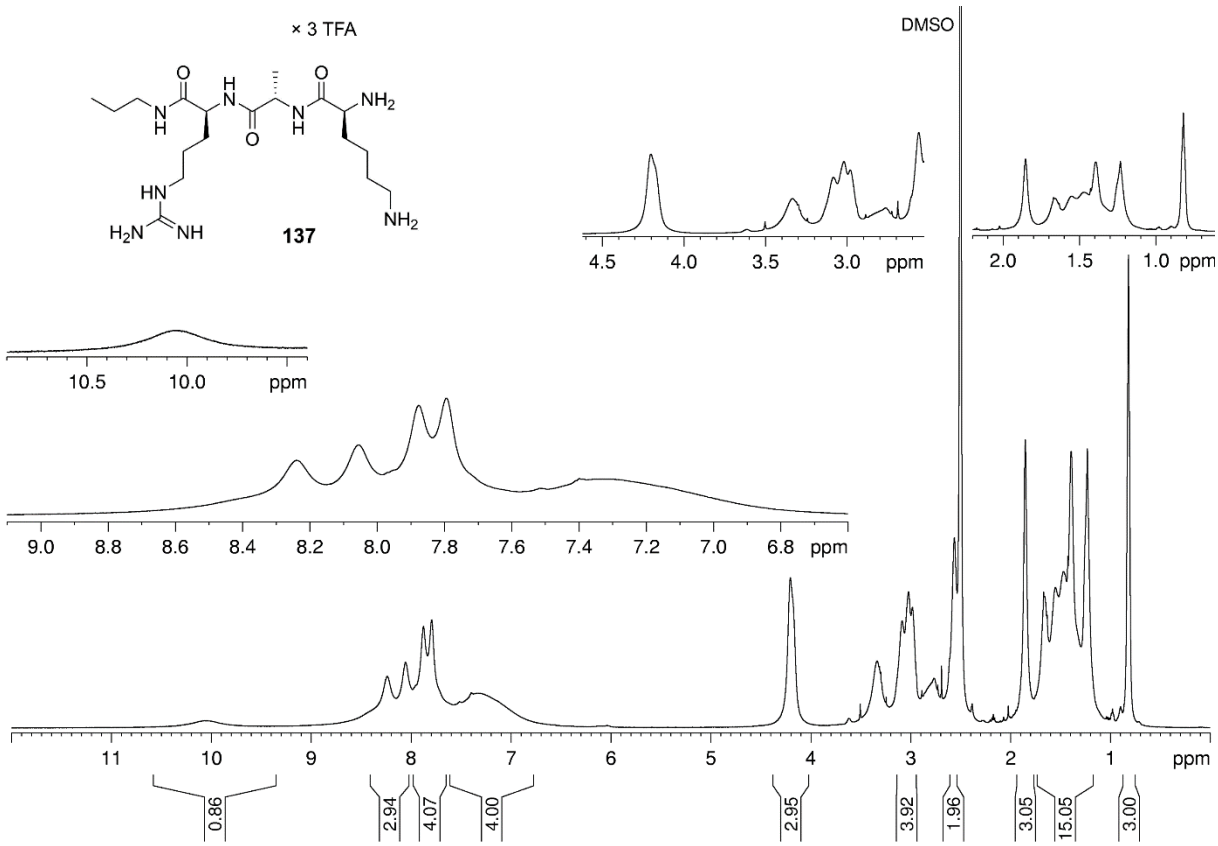
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **135**



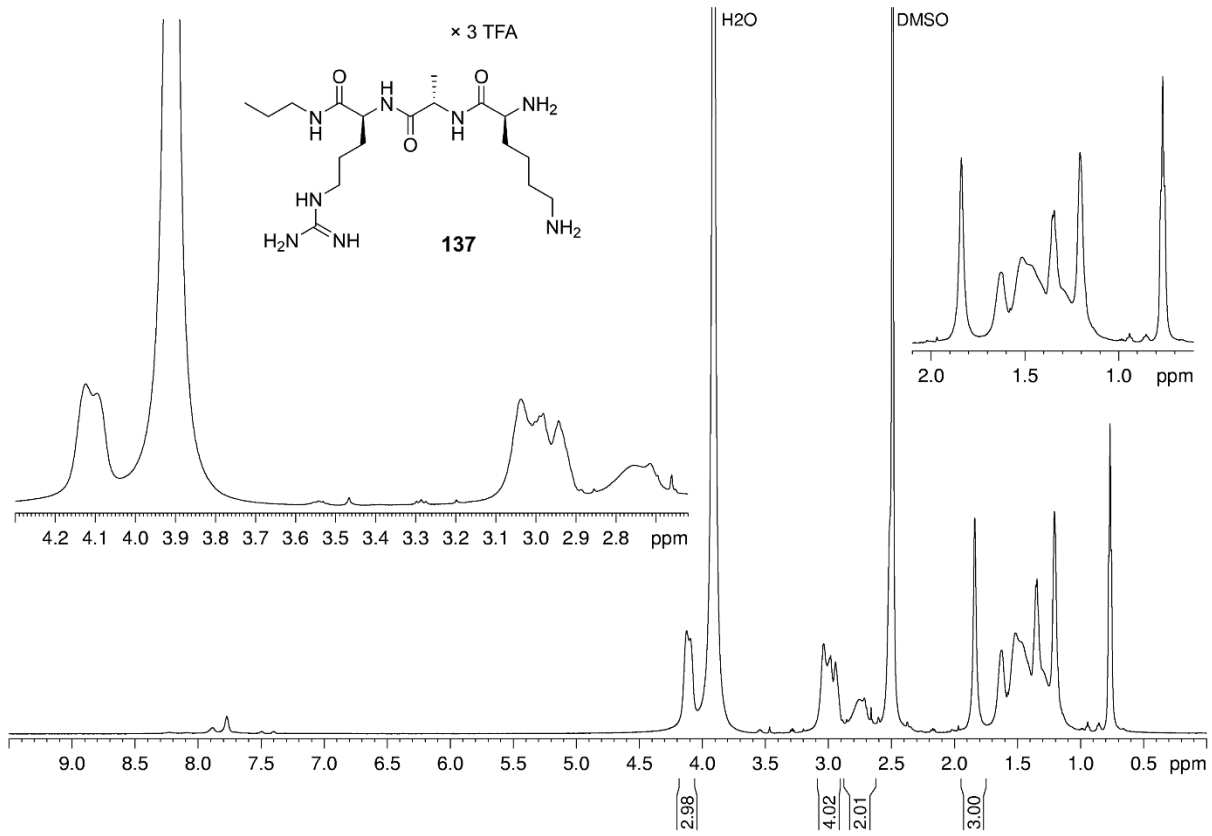
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **135**



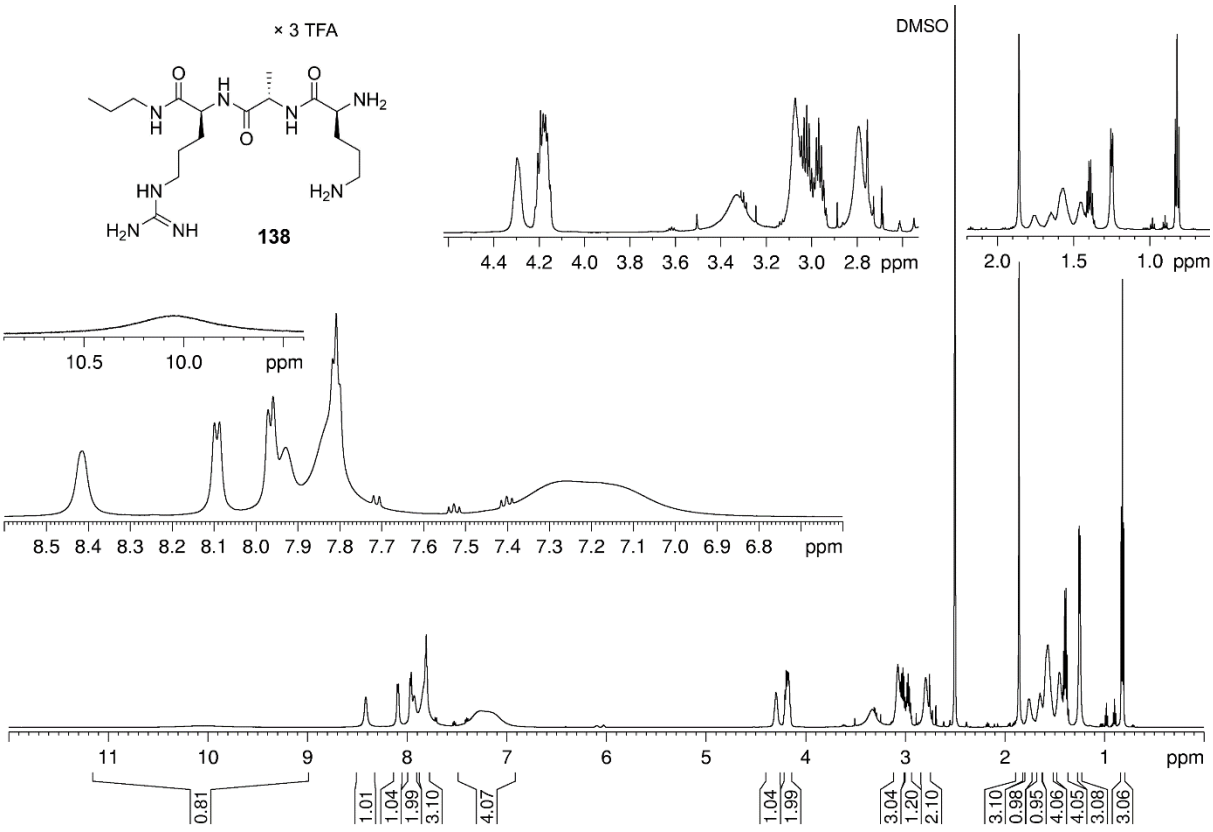
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **135**



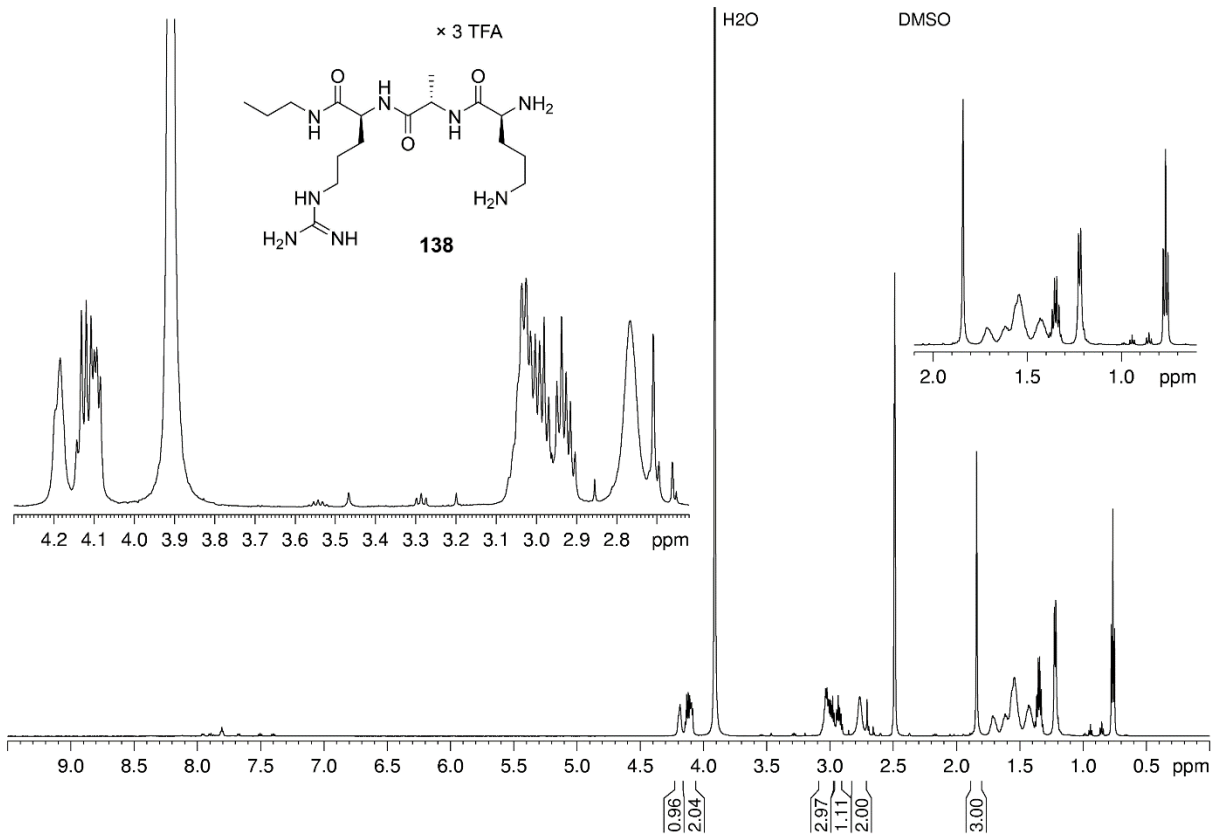
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **137**



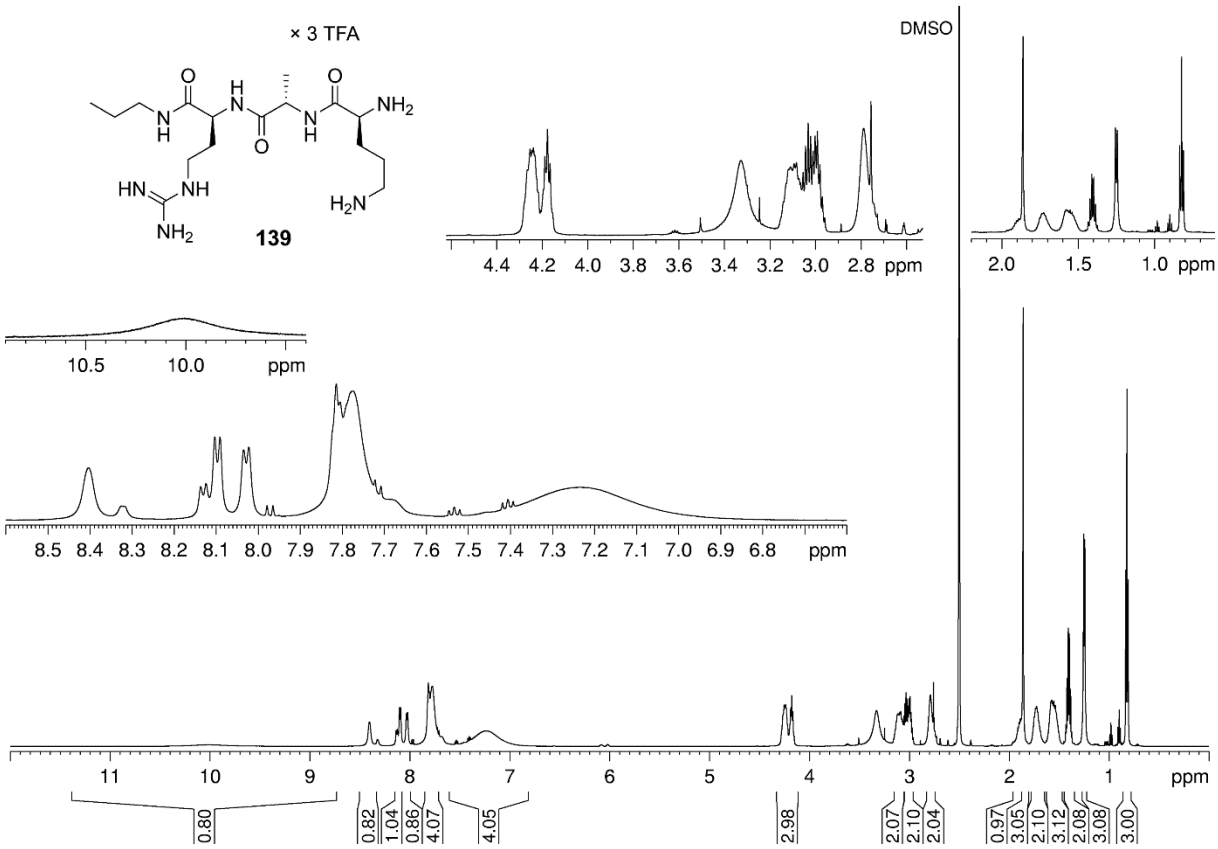
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **137**



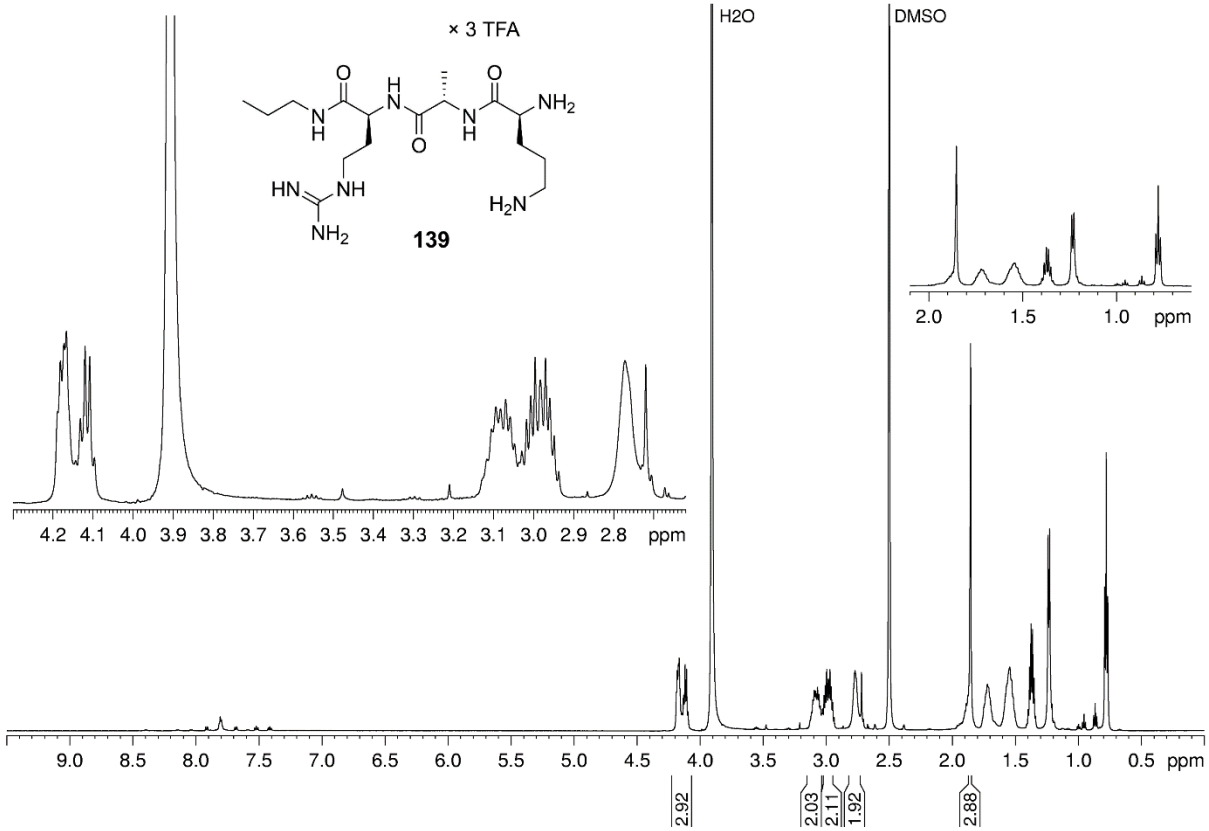
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **138**



^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **138**

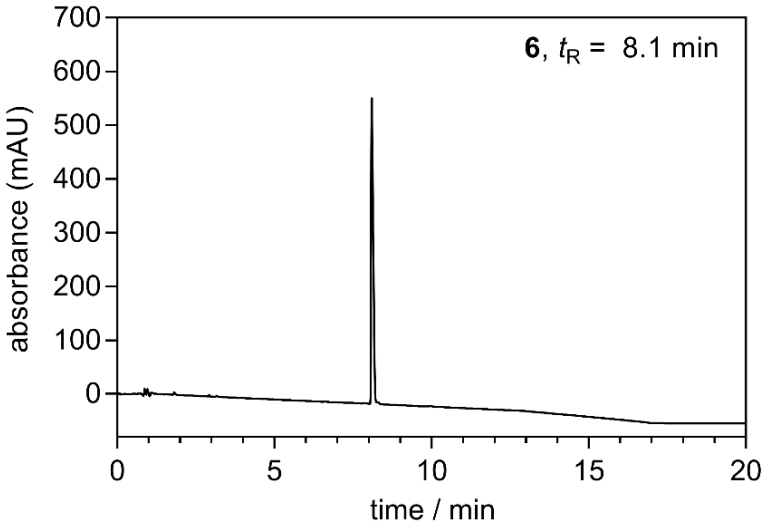


^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **139**

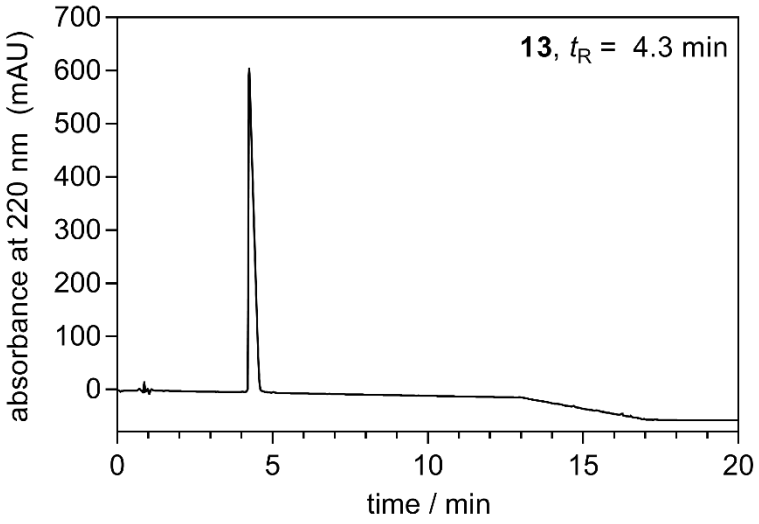


^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **139**

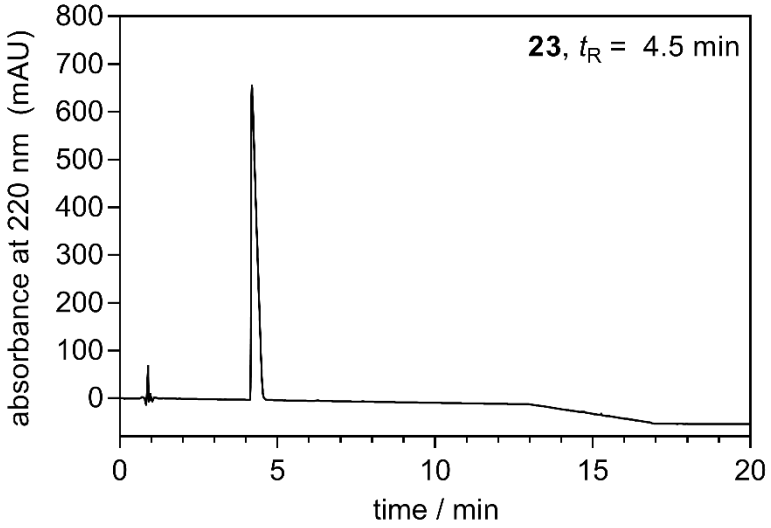
RP-HPLC chromatograms of compounds 6, 13, 23-25, 31, 35, 40, 43 53-60,63-65, 73-82, 85-99, 101-126, 134 and 135 (Chapter 2)



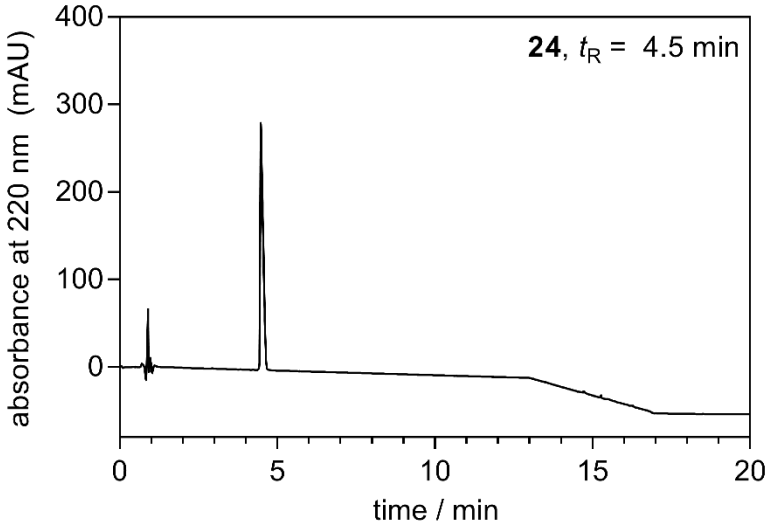
Chromatogram of the RP-HPLC analysis (purity control) of compound **6**



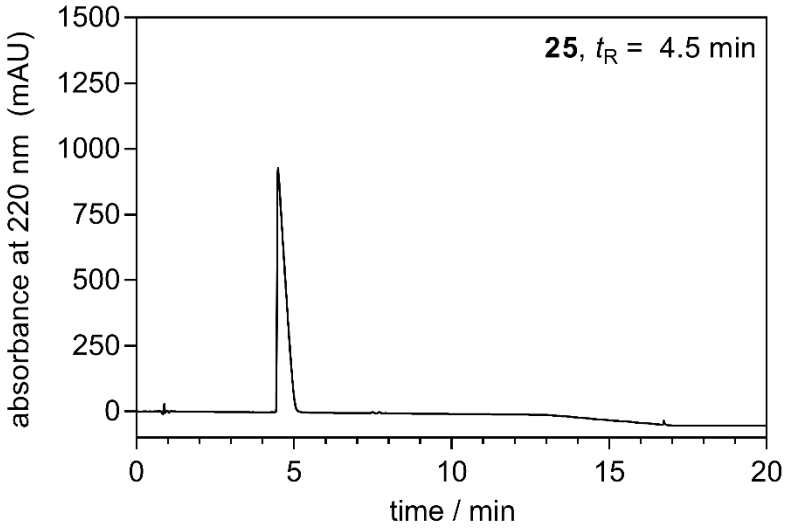
Chromatogram of the RP-HPLC analysis (purity control) of compound **13**



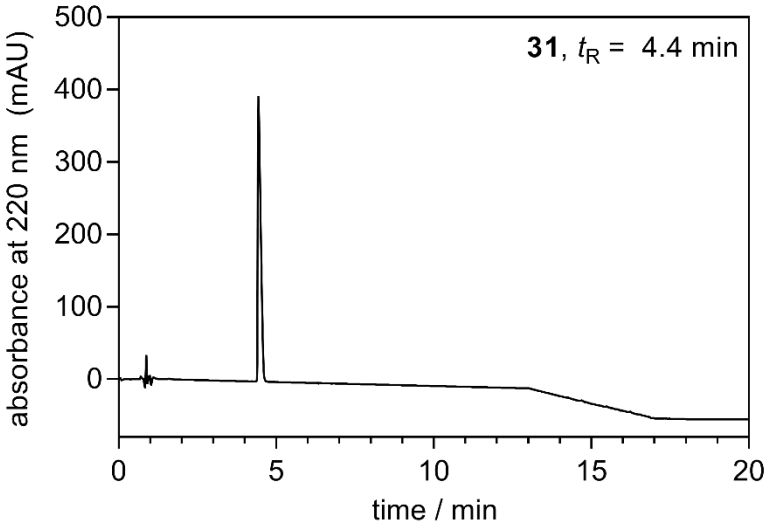
Chromatogram of the RP-HPLC analysis (purity control) of compound **23**



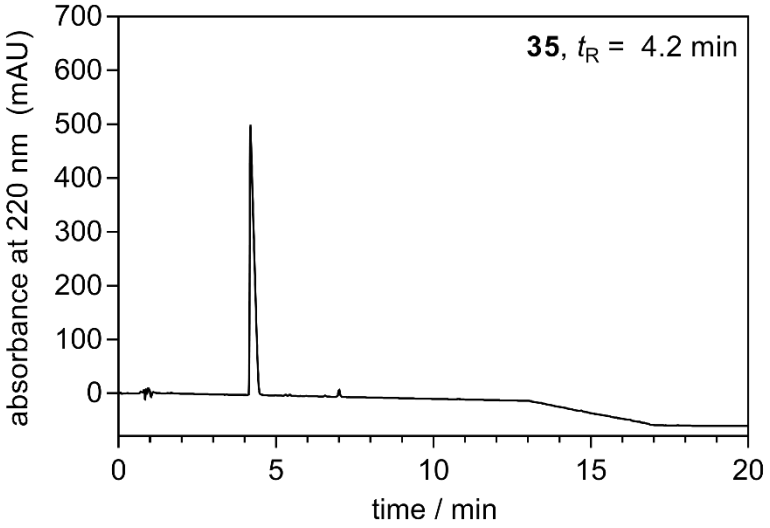
Chromatogram of the RP-HPLC analysis (purity control) of compound **24**



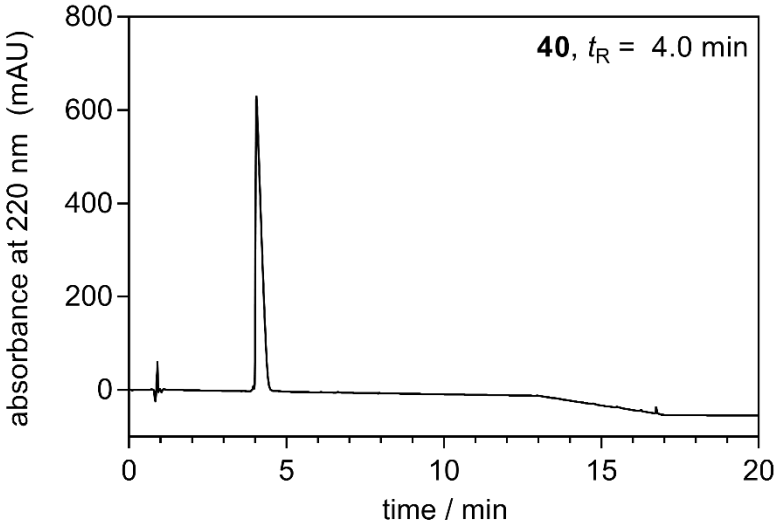
Chromatogram of the RP-HPLC analysis (purity control) of compound **25**



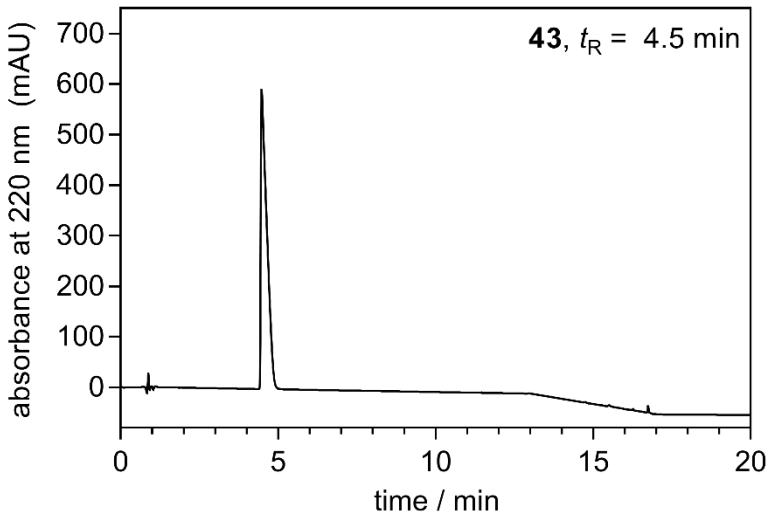
Chromatogram of the RP-HPLC analysis (purity control) of compound **31**



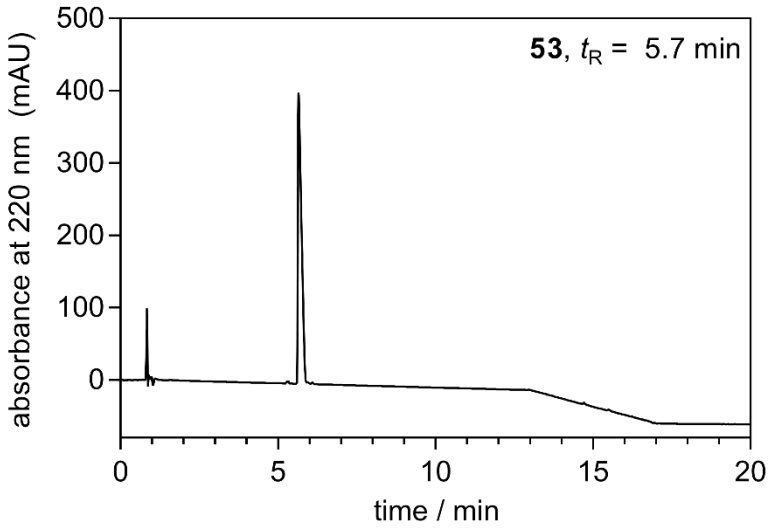
Chromatogram of the RP-HPLC analysis (purity control) of compound **35**



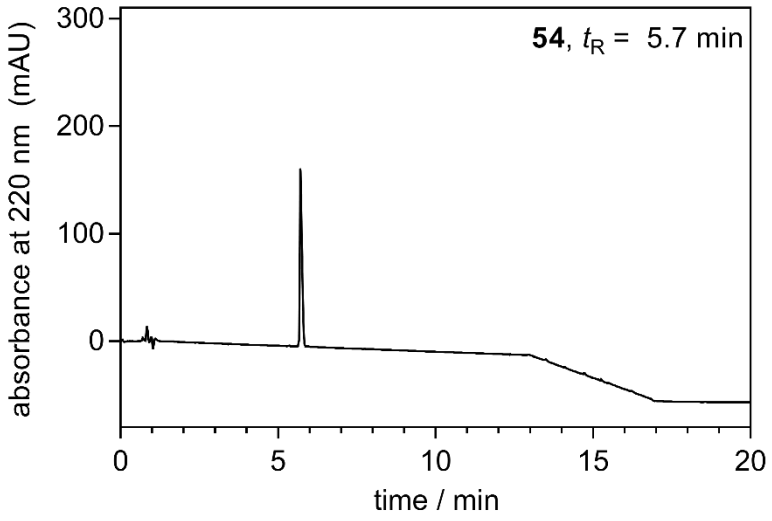
Chromatogram of the RP-HPLC analysis (purity control) of compound **40**



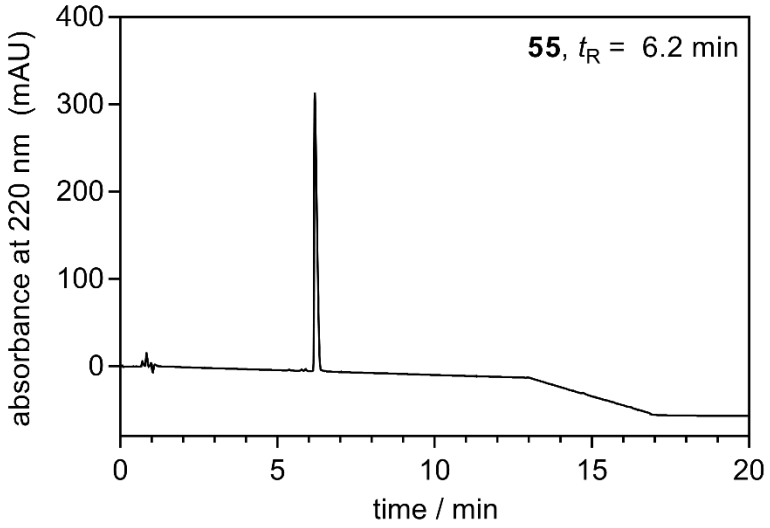
Chromatogram of the RP-HPLC analysis (purity control) of compound **43**



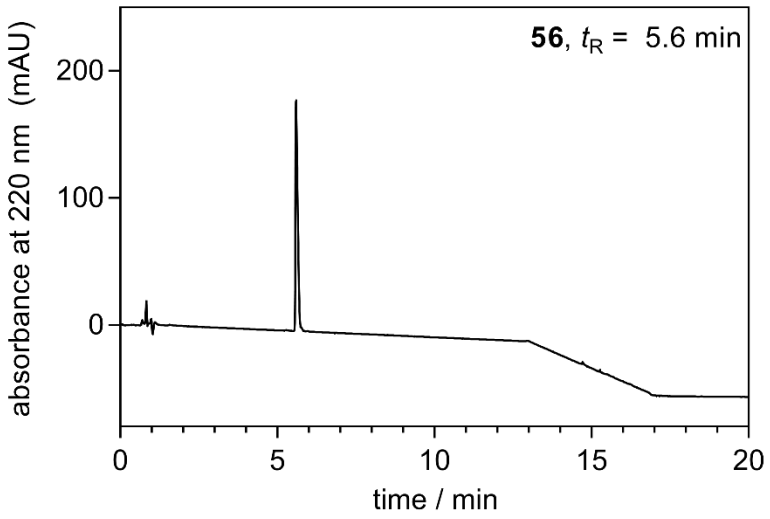
Chromatogram of the RP-HPLC analysis (purity control) of compound **53**



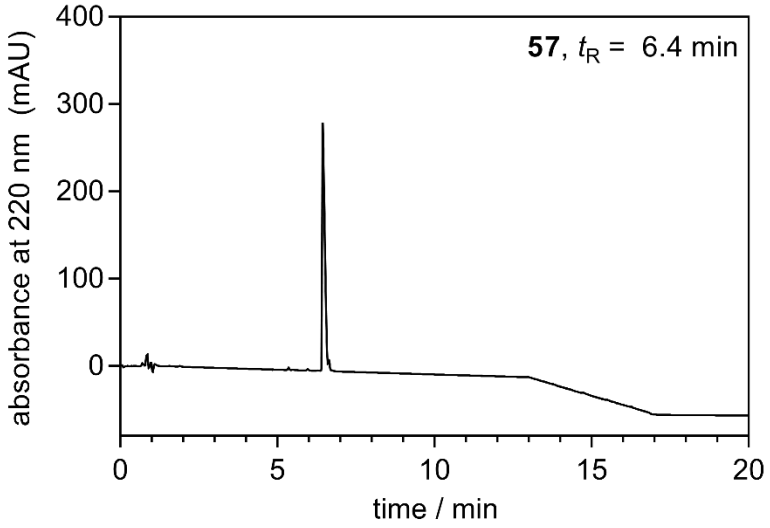
Chromatogram of the RP-HPLC analysis (purity control) of compound **54**



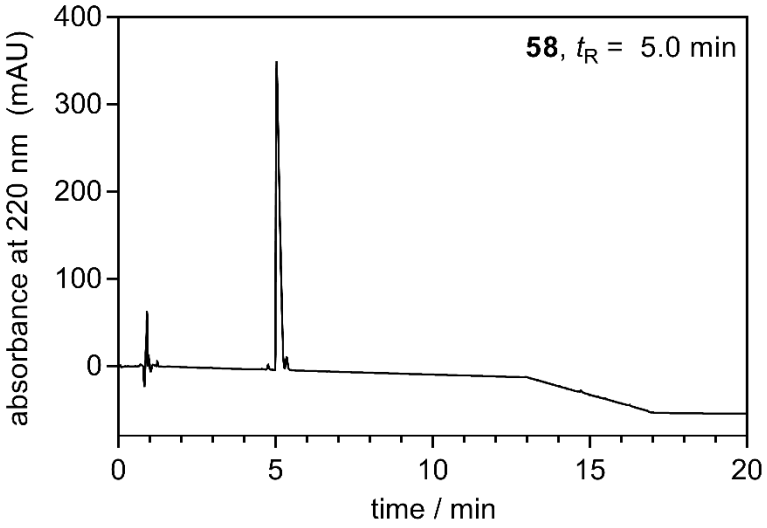
Chromatogram of the RP-HPLC analysis (purity control) of compound **55**



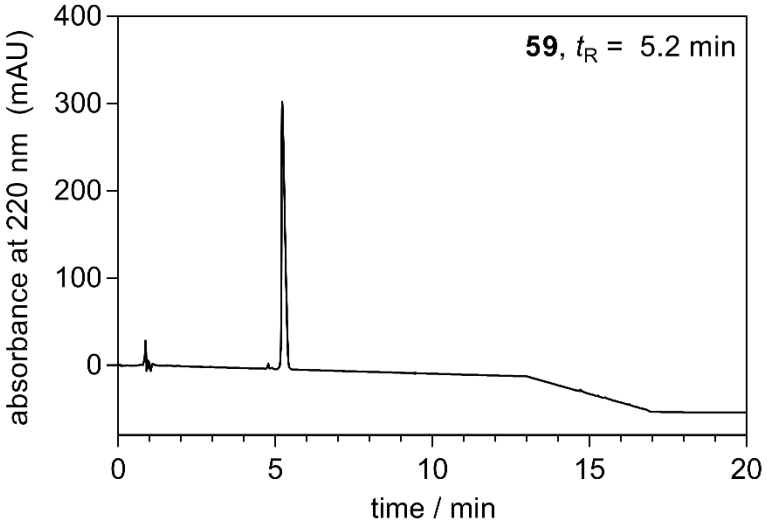
Chromatogram of the RP-HPLC analysis (purity control) of compound **56**



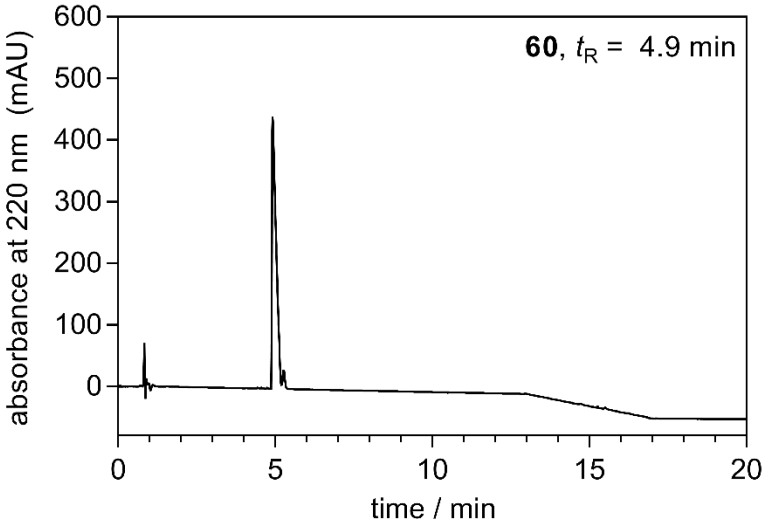
Chromatogram of the RP-HPLC analysis (purity control) of compound **57**



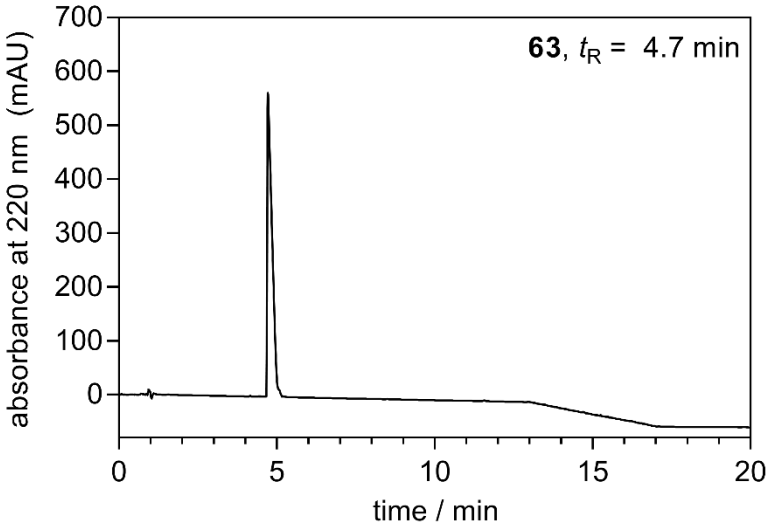
Chromatogram of the RP-HPLC analysis (purity control) of compound **58**



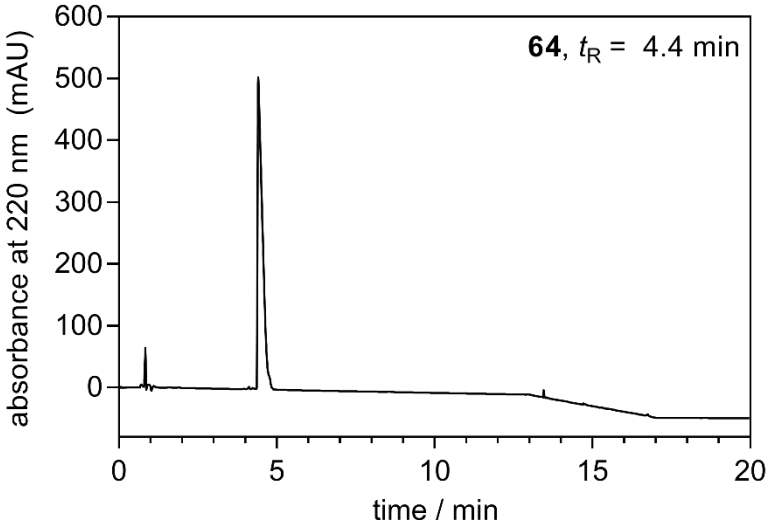
Chromatogram of the RP-HPLC analysis (purity control) of compound **59**



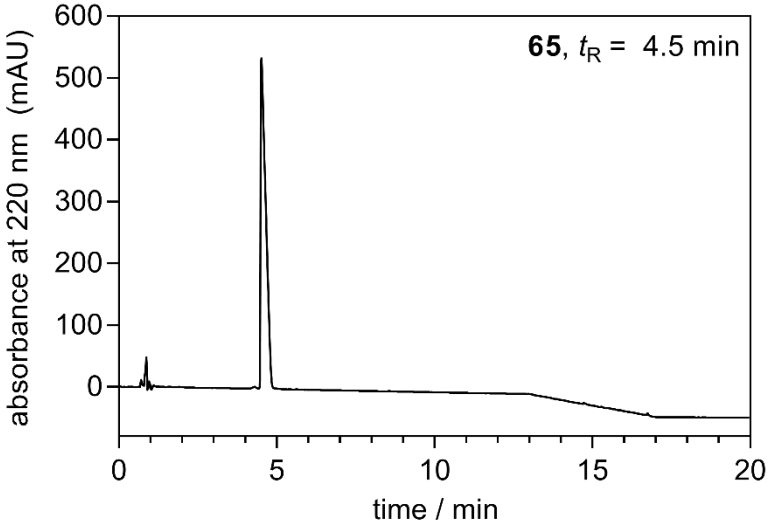
Chromatogram of the RP-HPLC analysis (purity control) of compound **60**



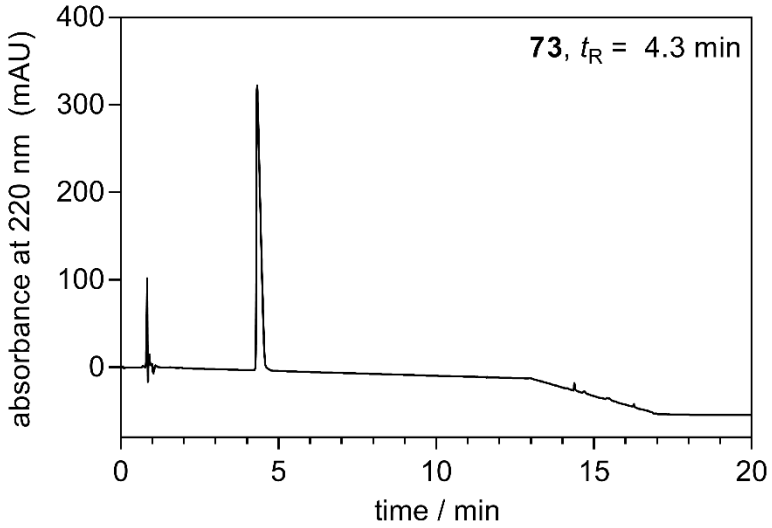
Chromatogram of the RP-HPLC analysis (purity control) of compound **63**



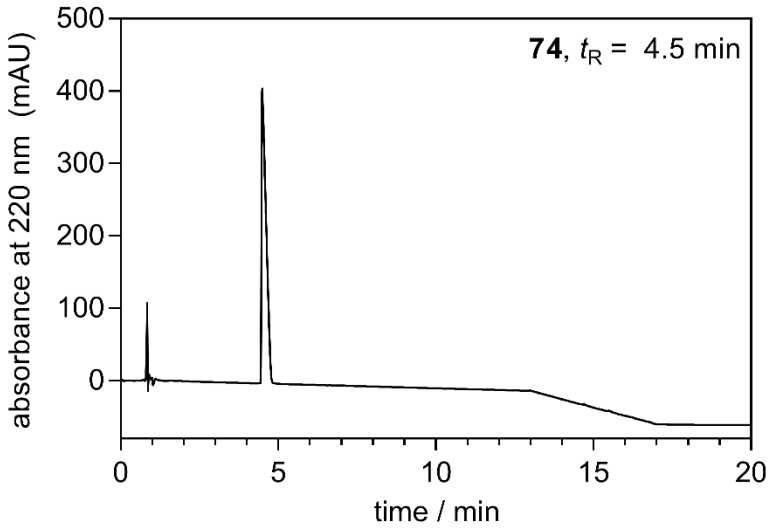
Chromatogram of the RP-HPLC analysis (purity control) of compound **64**



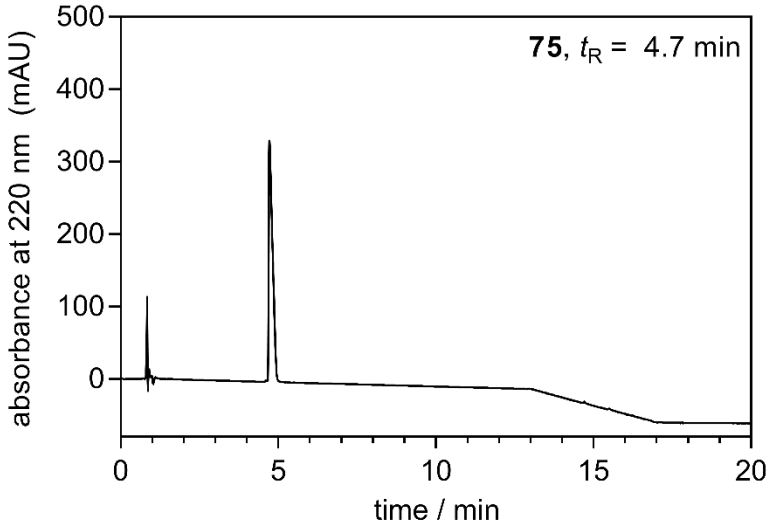
Chromatogram of the RP-HPLC analysis (purity control) of compound **65**



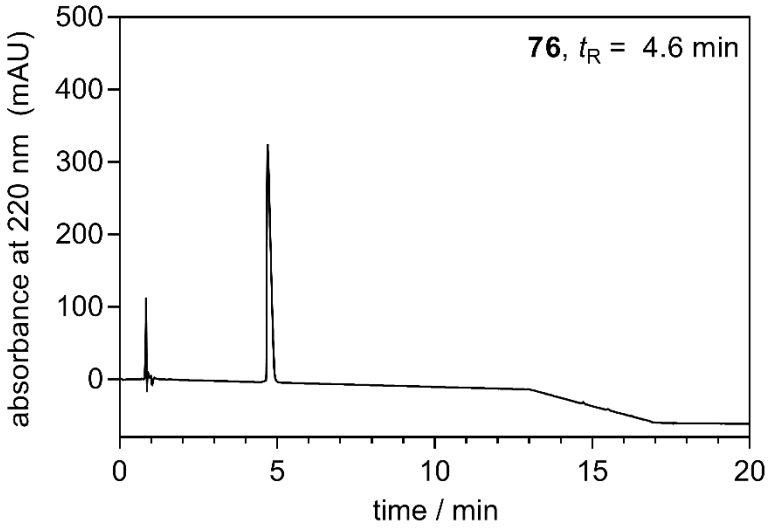
Chromatogram of the RP-HPLC analysis (purity control) of compound **73**



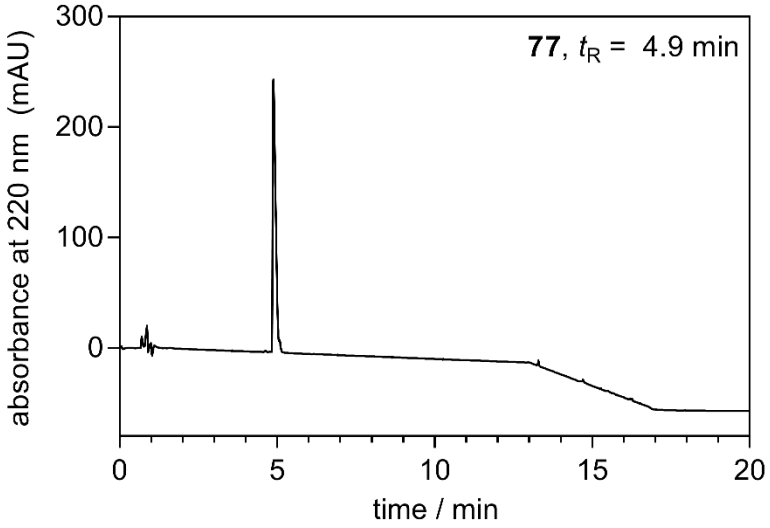
Chromatogram of the RP-HPLC analysis (purity control) of compound **74**



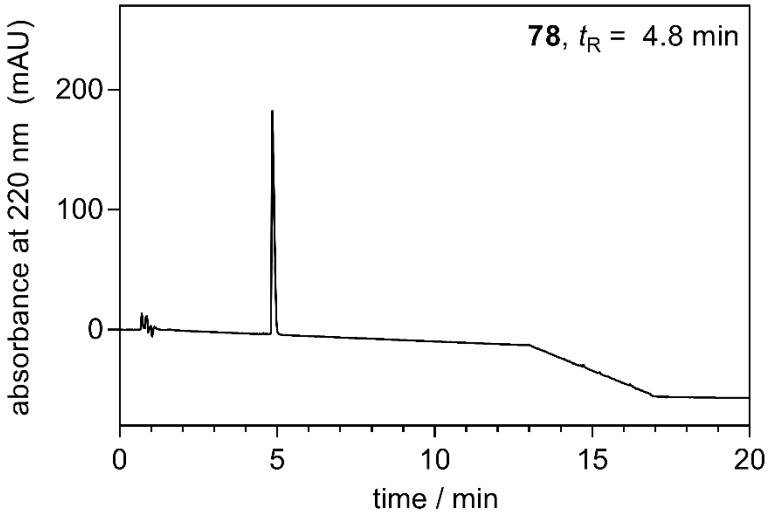
Chromatogram of the RP-HPLC analysis (purity control) of compound **75**



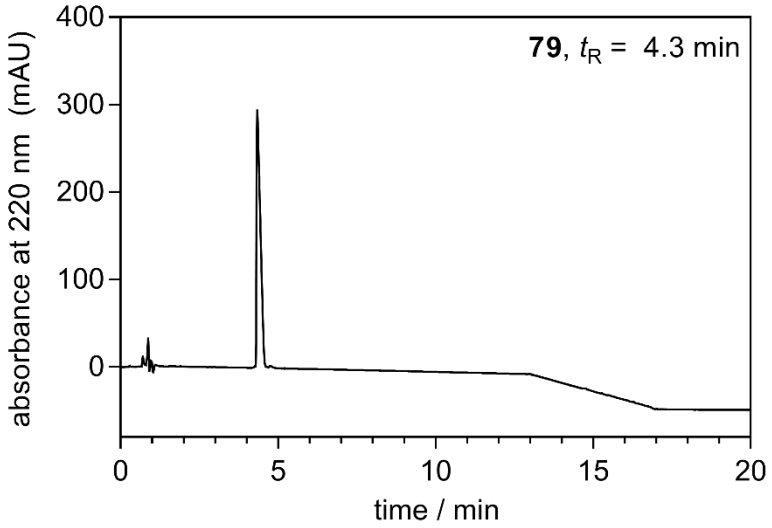
Chromatogram of the RP-HPLC analysis (purity control) of compound **76**



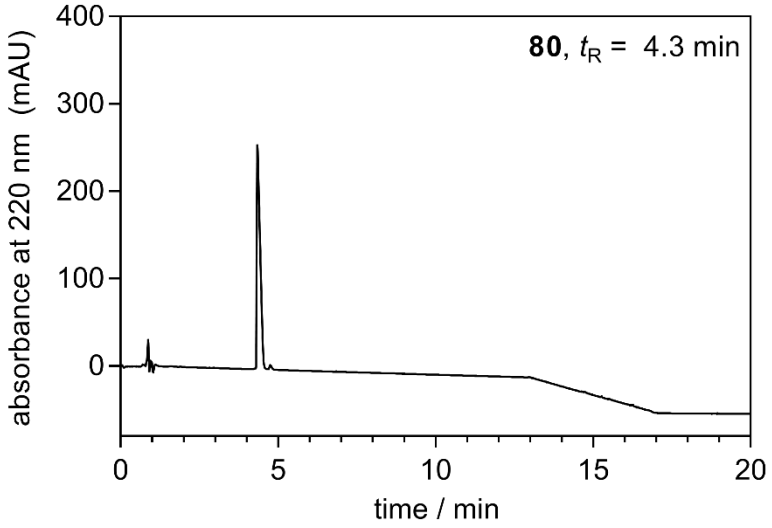
Chromatogram of the RP-HPLC analysis (purity control) of compound **77**



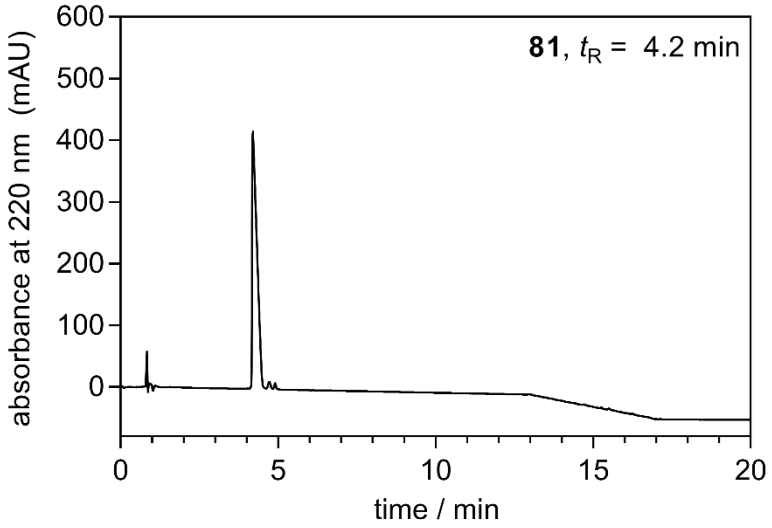
Chromatogram of the RP-HPLC analysis (purity control) of compound **78**



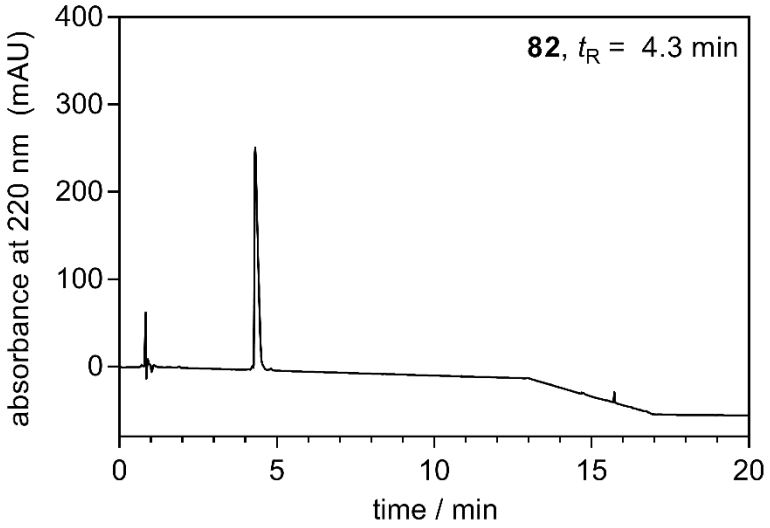
Chromatogram of the RP-HPLC analysis (purity control) of compound **79**



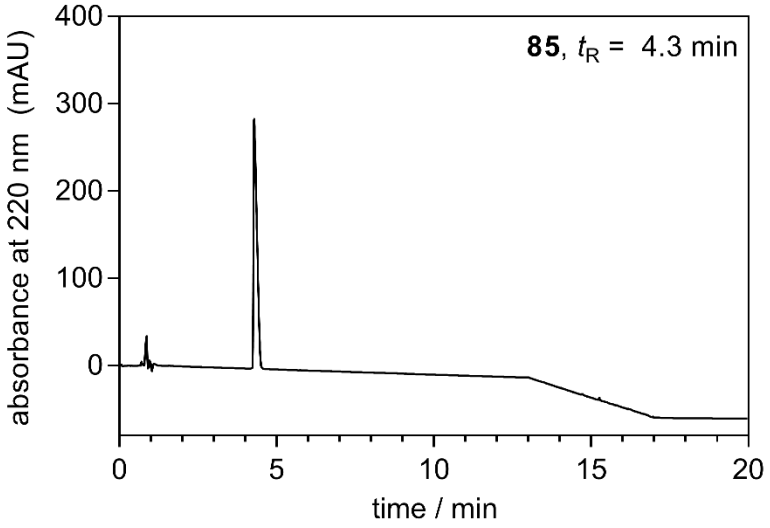
Chromatogram of the RP-HPLC analysis (purity control) of compound **80**



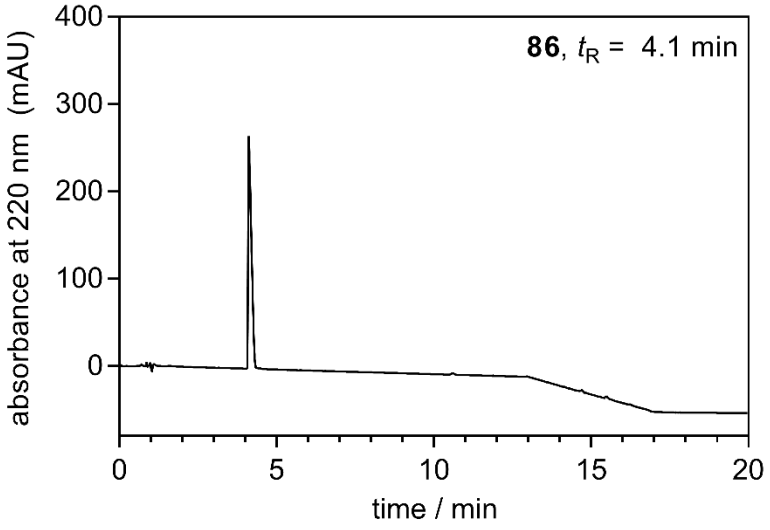
Chromatogram of the RP-HPLC analysis (purity control) of compound **81**



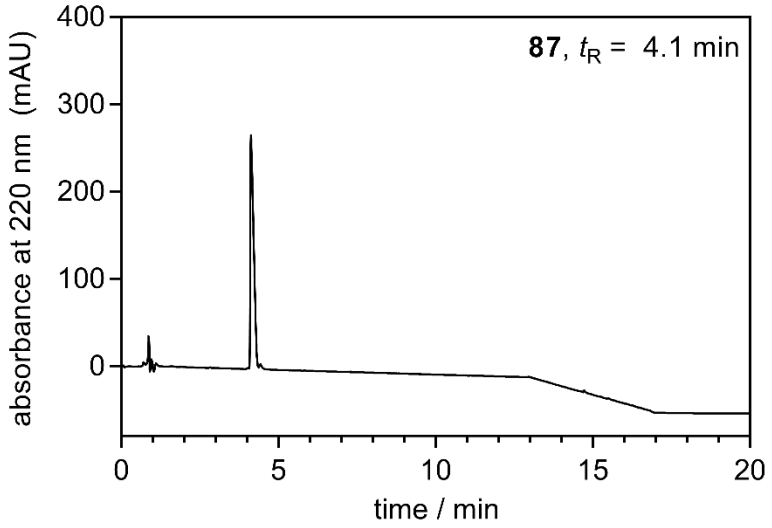
Chromatogram of the RP-HPLC analysis (purity control) of compound **82**



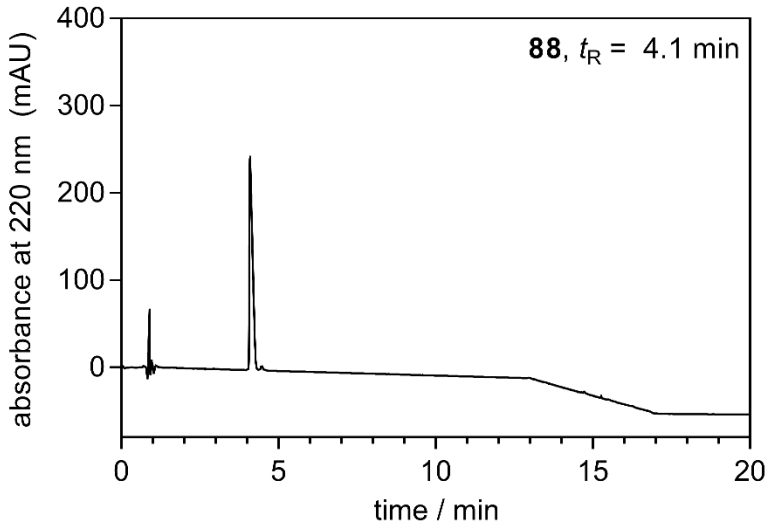
Chromatogram of the RP-HPLC analysis (purity control) of compound **85**



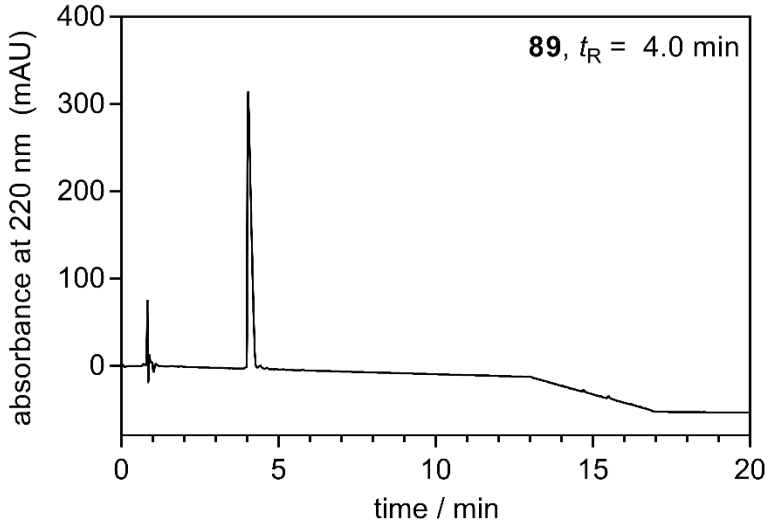
Chromatogram of the RP-HPLC analysis (purity control) of compound **86**



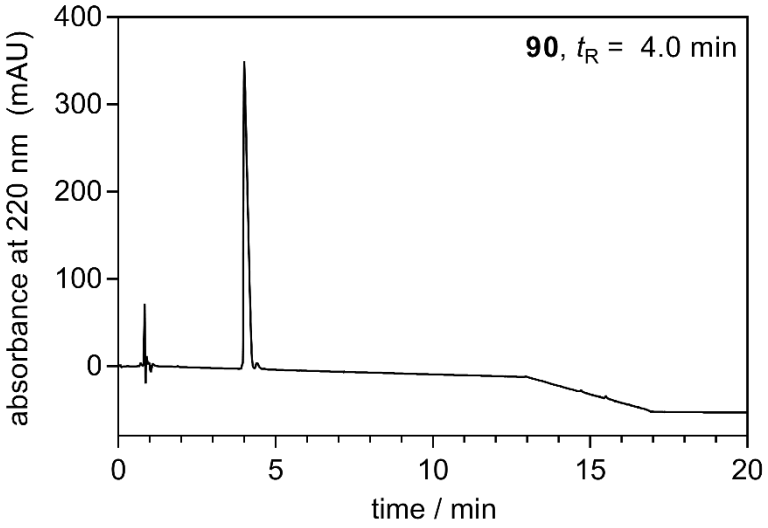
Chromatogram of the RP-HPLC analysis (purity control) of compound **87**



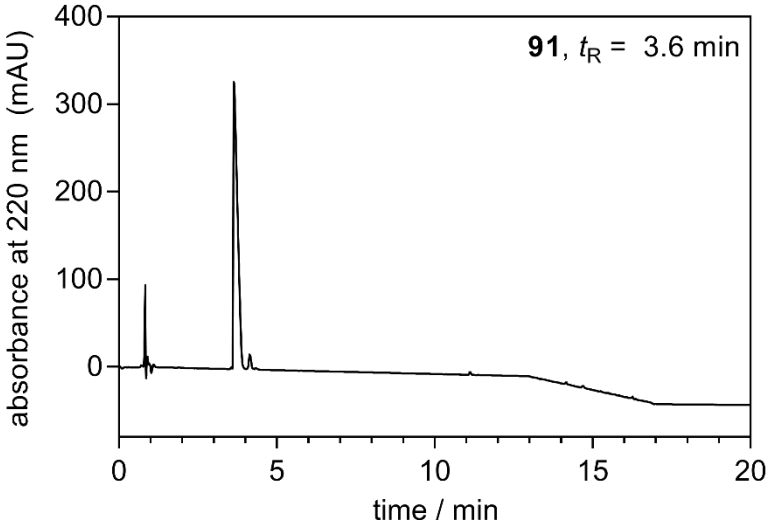
Chromatogram of the RP-HPLC analysis (purity control) of compound **88**



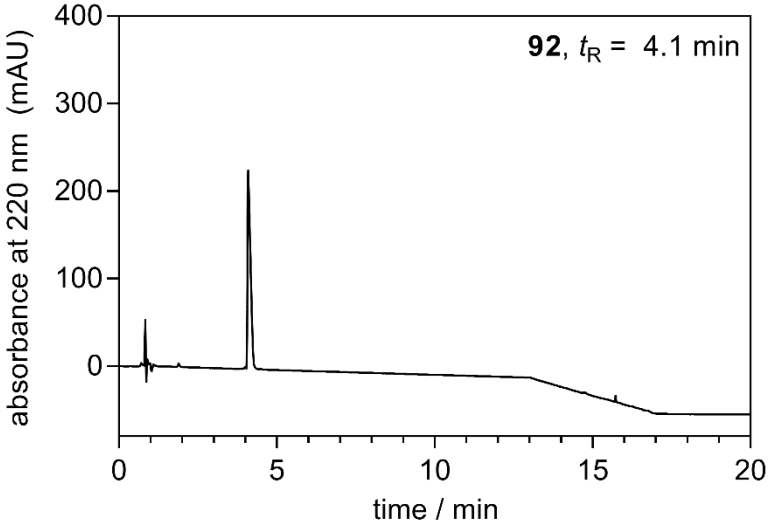
Chromatogram of the RP-HPLC analysis (purity control) of compound **89**



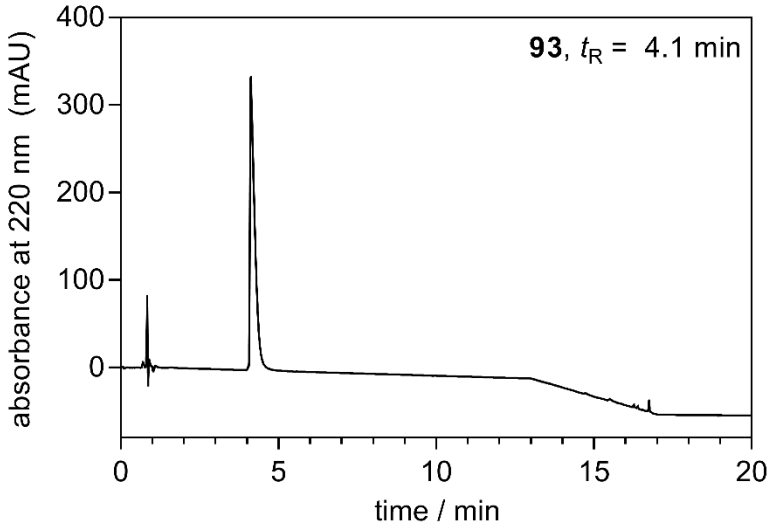
Chromatogram of the RP-HPLC analysis (purity control) of compound **90**



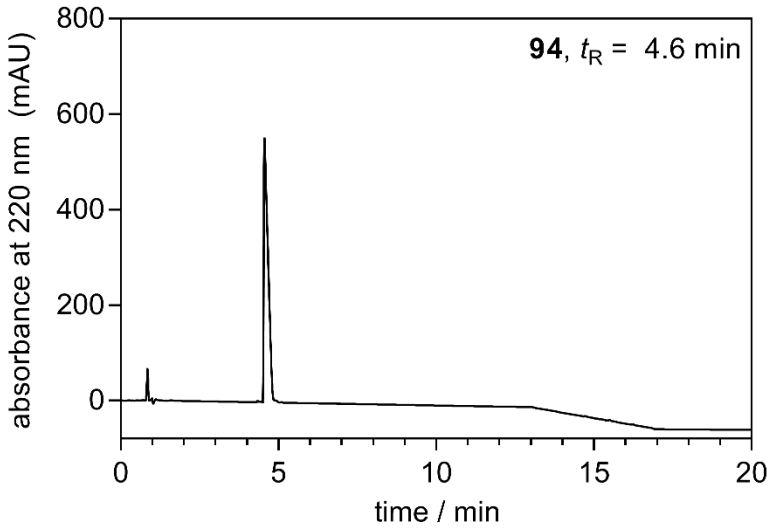
Chromatogram of the RP-HPLC analysis (purity control) of compound **91**



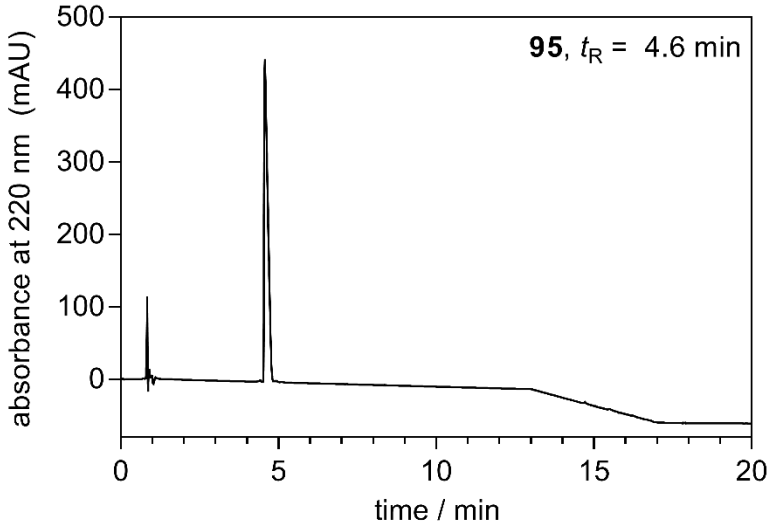
Chromatogram of the RP-HPLC analysis (purity control) of compound **92**



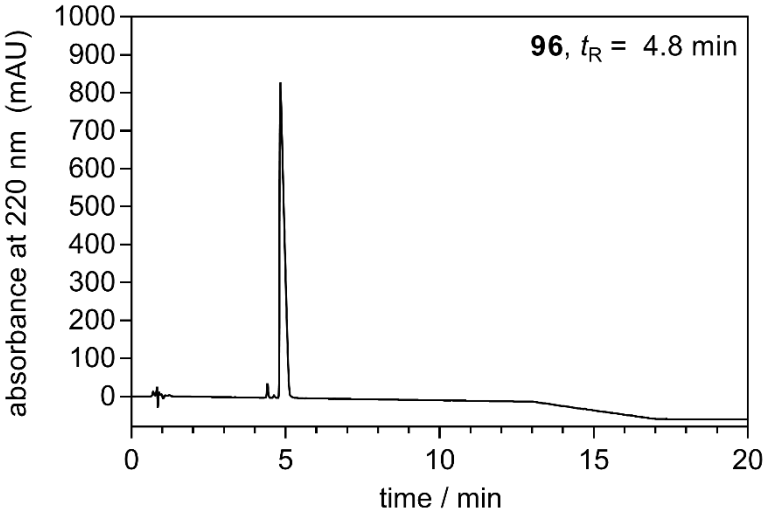
Chromatogram of the RP-HPLC analysis (purity control) of compound **93**



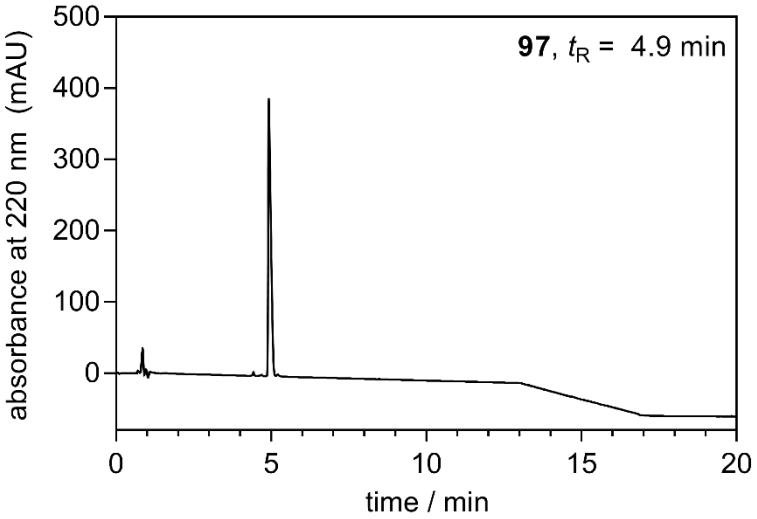
Chromatogram of the RP-HPLC analysis (purity control) of compound **94**



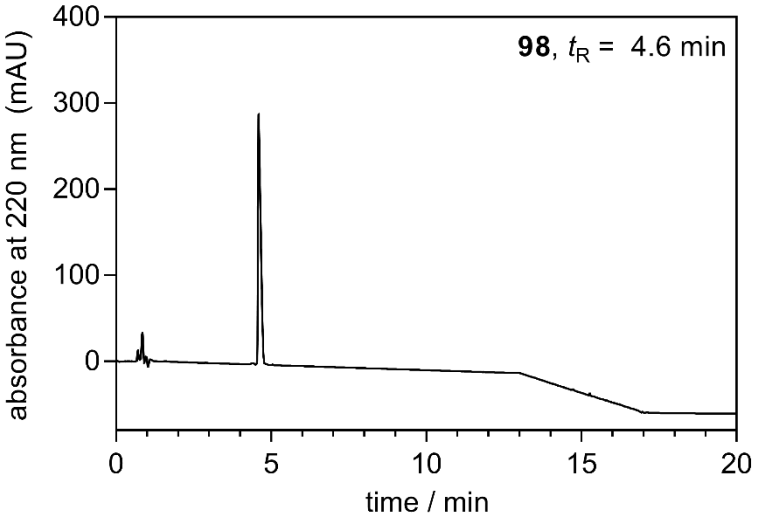
Chromatogram of the RP-HPLC analysis (purity control) of compound **95**



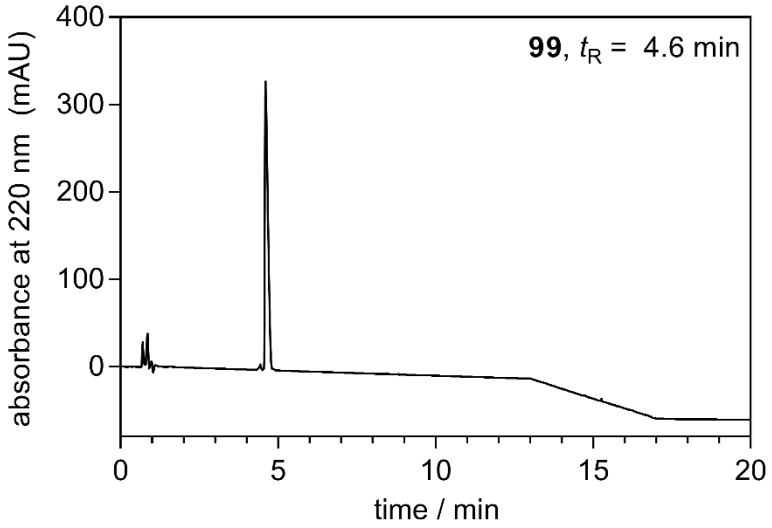
Chromatogram of the RP-HPLC analysis (purity control) of compound **96**



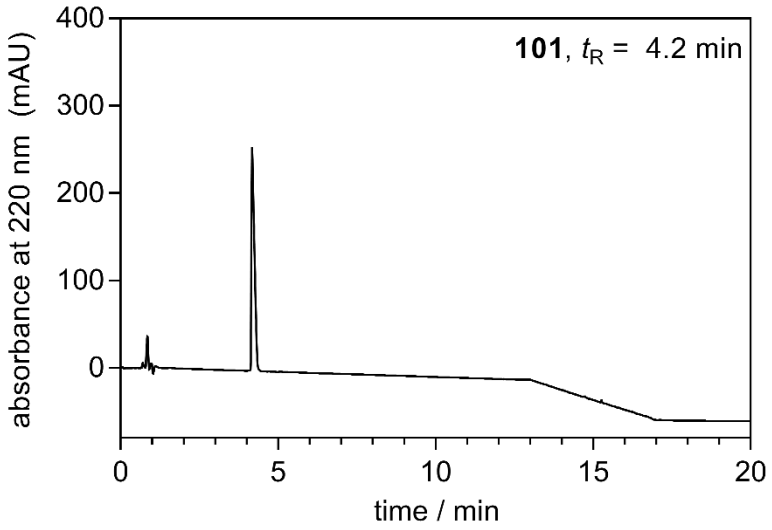
Chromatogram of the RP-HPLC analysis (purity control) of compound **97**



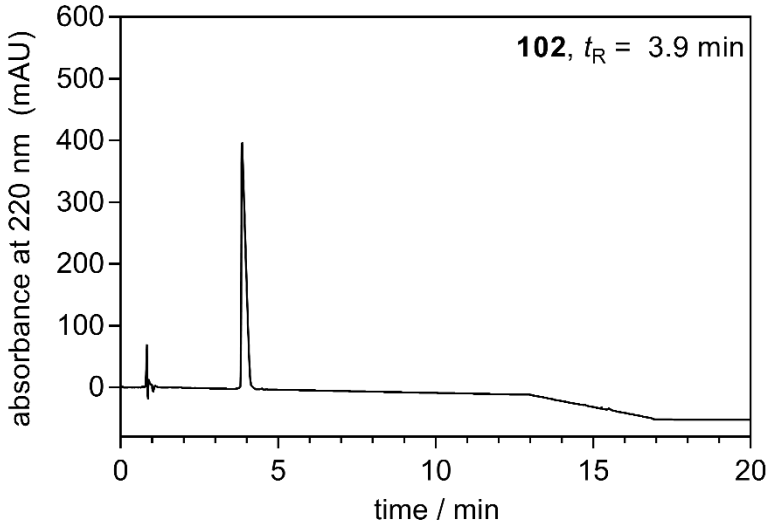
Chromatogram of the RP-HPLC analysis (purity control) of compound **98**



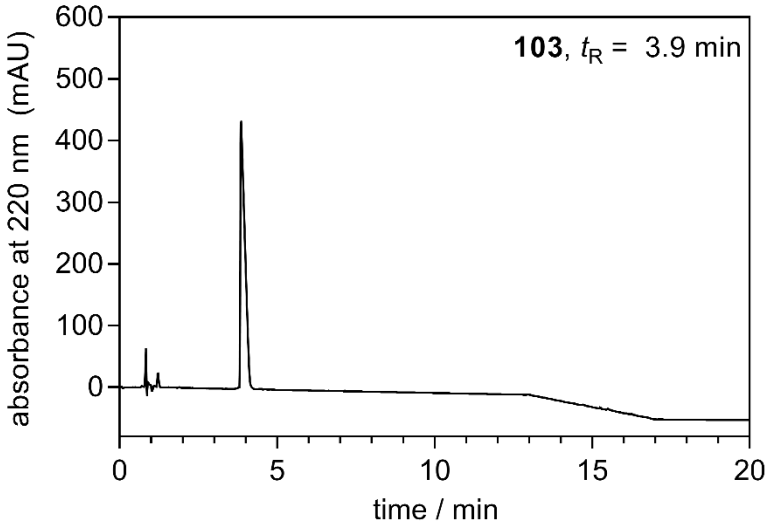
Chromatogram of the RP-HPLC analysis (purity control) of compound **99**



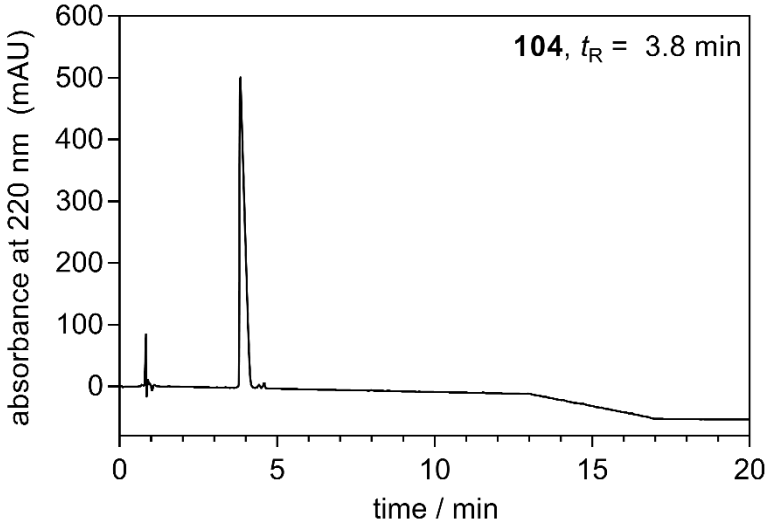
Chromatogram of the RP-HPLC analysis (purity control) of compound **101**



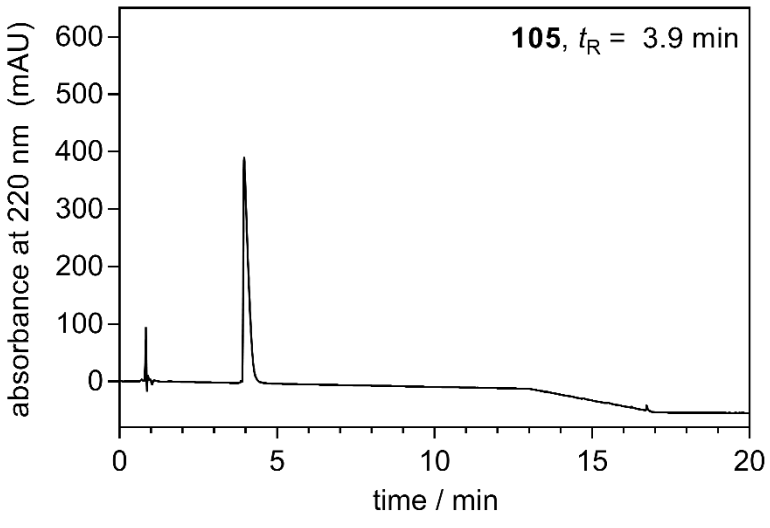
Chromatogram of the RP-HPLC analysis (purity control) of compound **102**



Chromatogram of the RP-HPLC analysis (purity control) of compound **103**



Chromatogram of the RP-HPLC analysis (purity control) of compound **104**



Chromatogram of the RP-HPLC analysis (purity control) of compound **105**

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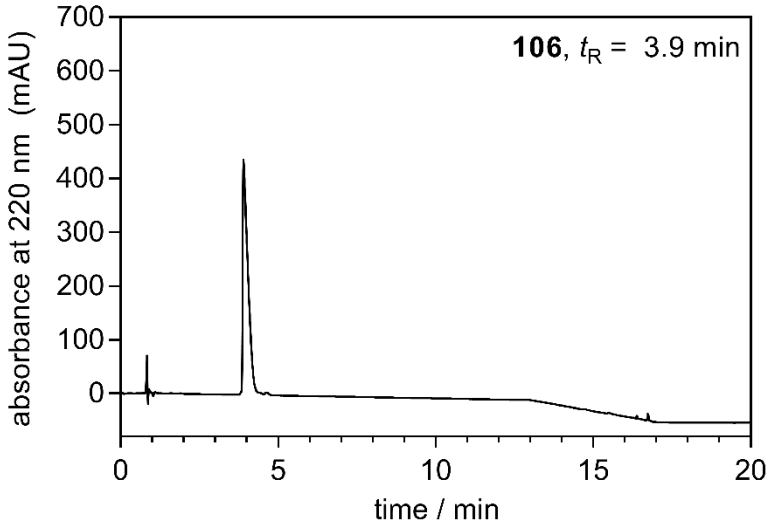
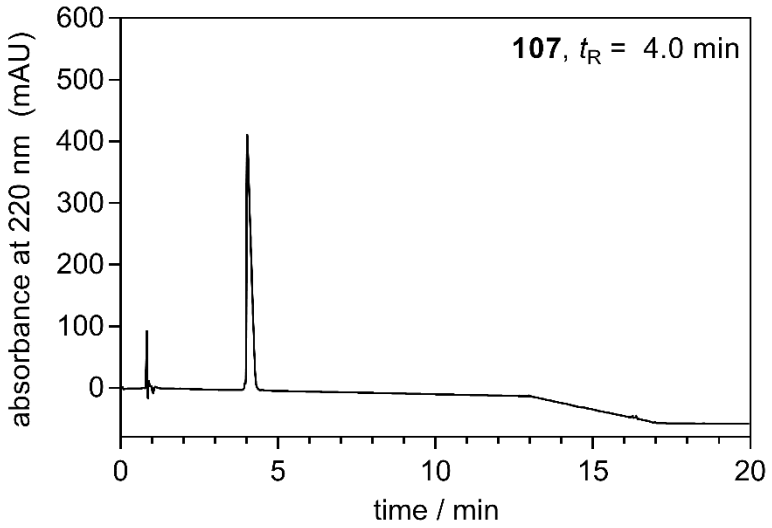
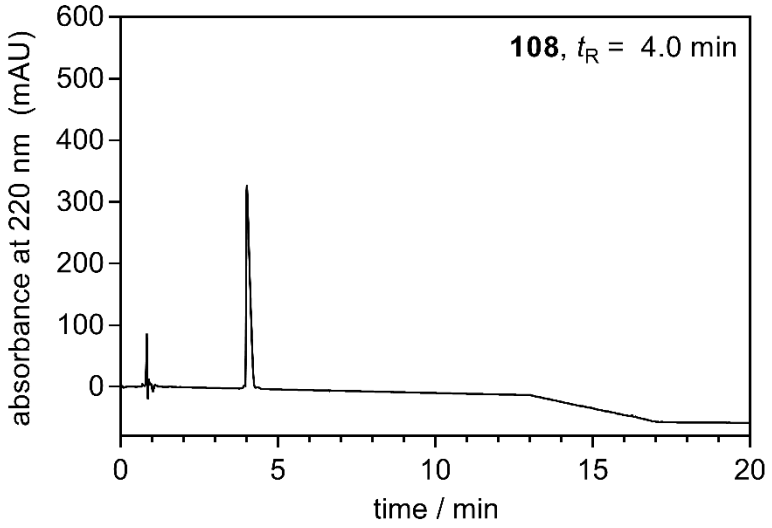


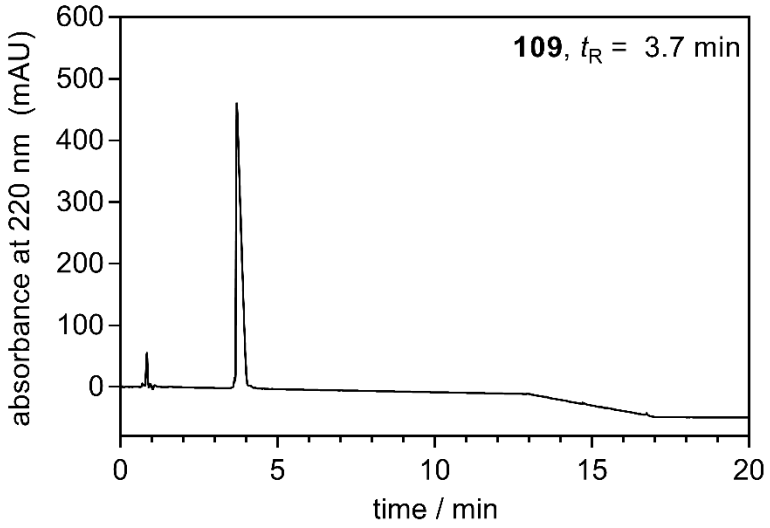
Chromatogram of the RP-HPLC analysis (purity control) of compound **106**



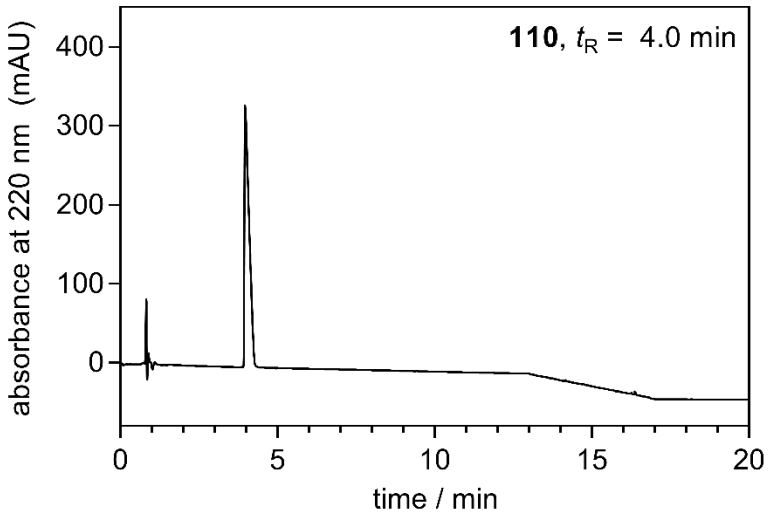
Chromatogram of the RP-HPLC analysis (purity control) of compound **107**



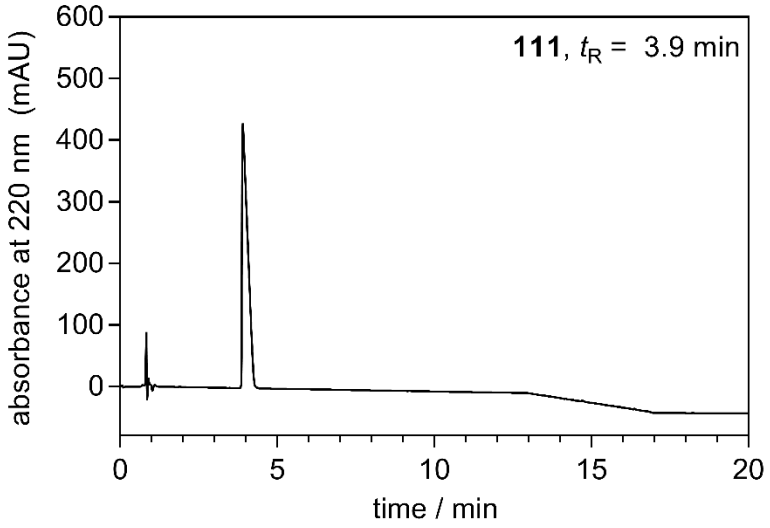
Chromatogram of the RP-HPLC analysis (purity control) of compound **108**



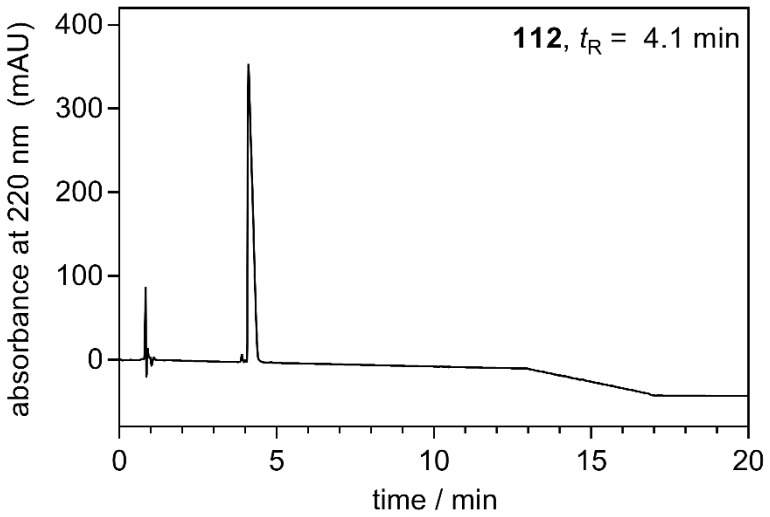
Chromatogram of the RP-HPLC analysis (purity control) of compound **109**



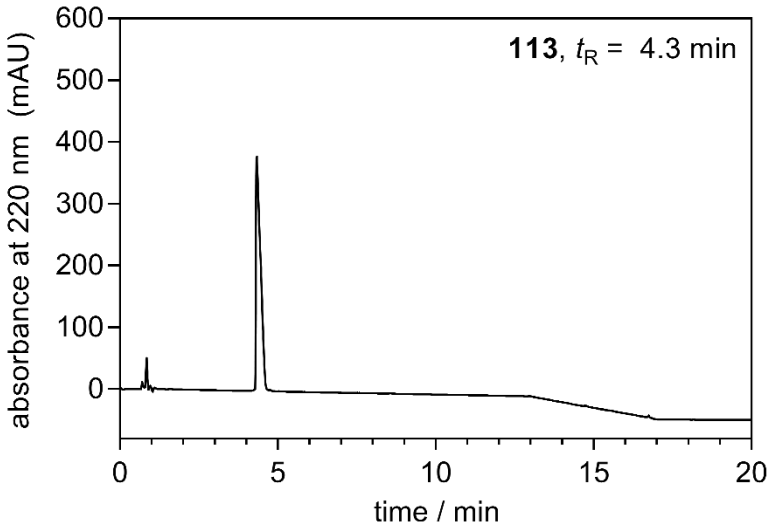
Chromatogram of the RP-HPLC analysis (purity control) of compound **110**



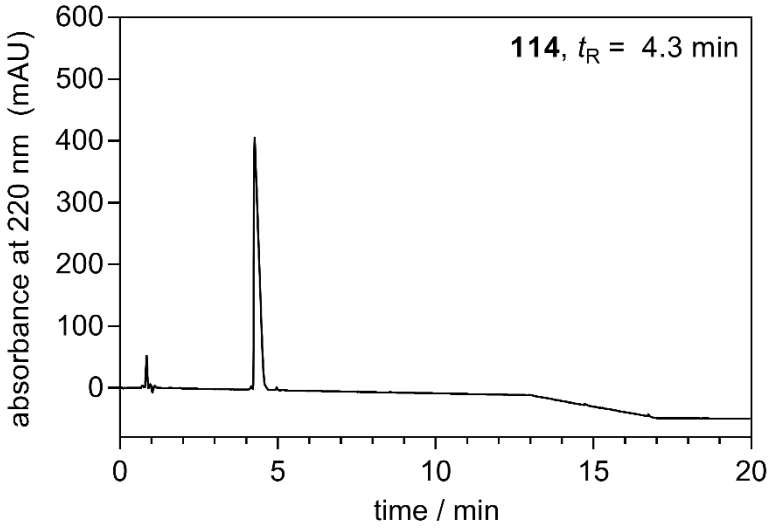
Chromatogram of the RP-HPLC analysis (purity control) of compound **111**



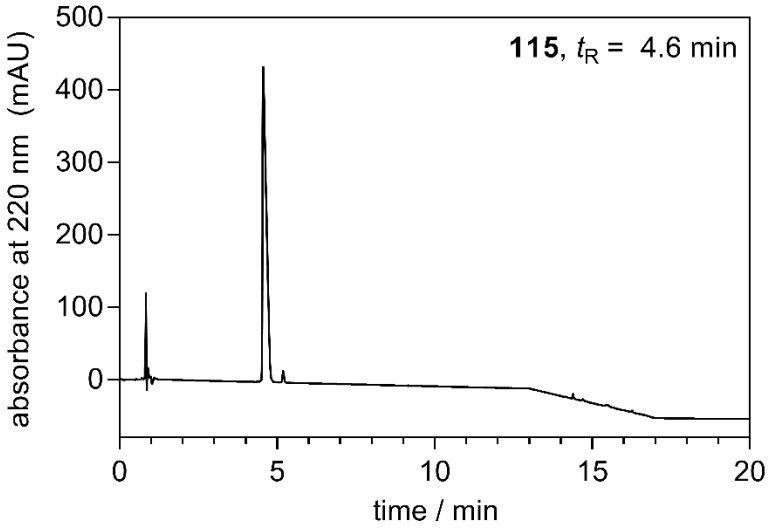
Chromatogram of the RP-HPLC analysis (purity control) of compound **112**



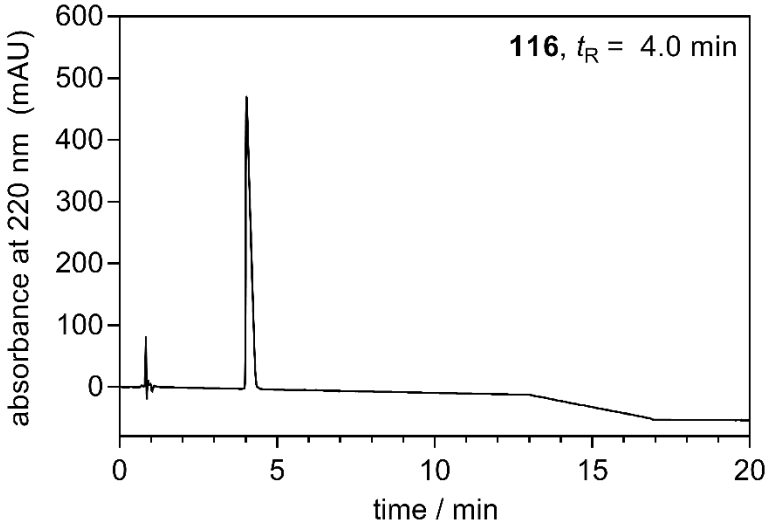
Chromatogram of the RP-HPLC analysis (purity control) of compound **113**



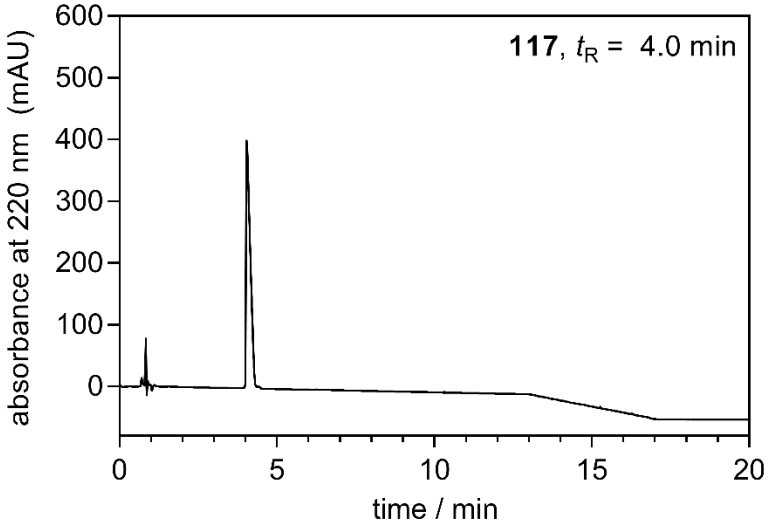
Chromatogram of the RP-HPLC analysis (purity control) of compound **114**



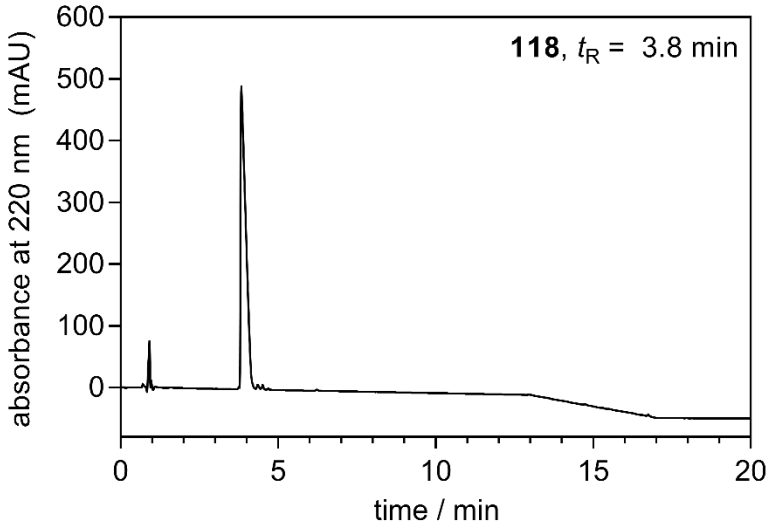
Chromatogram of the RP-HPLC analysis (purity control) of compound **115**



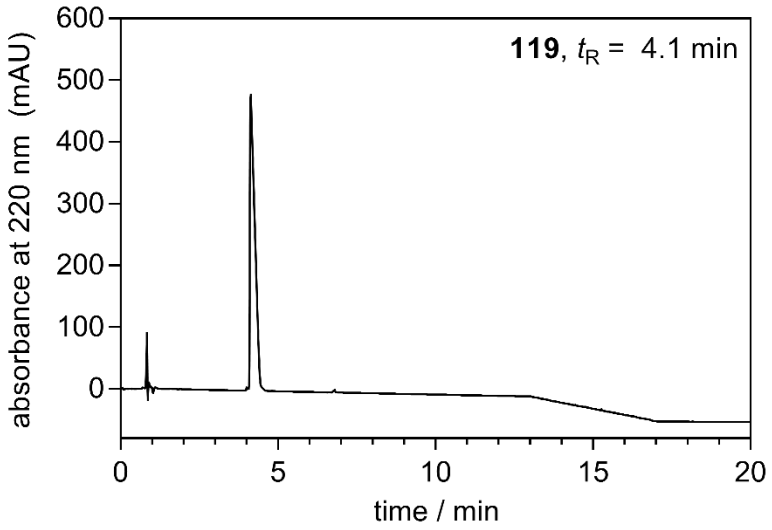
Chromatogram of the RP-HPLC analysis (purity control) of compound **116**



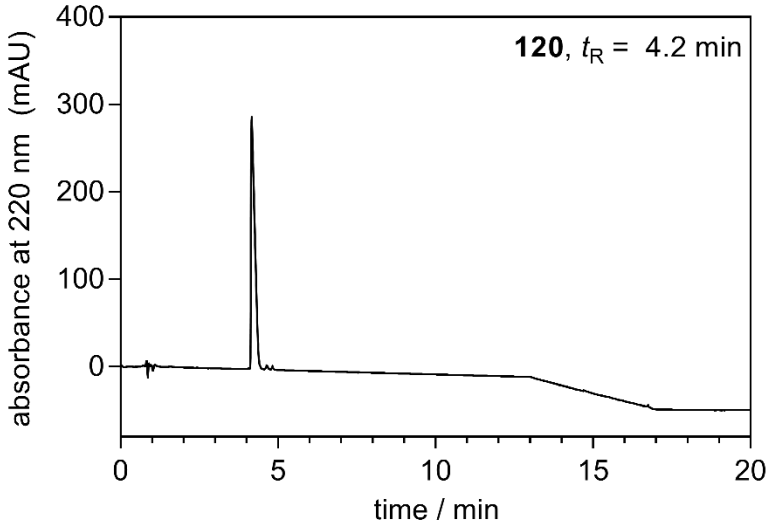
Chromatogram of the RP-HPLC analysis (purity control) of compound **117**



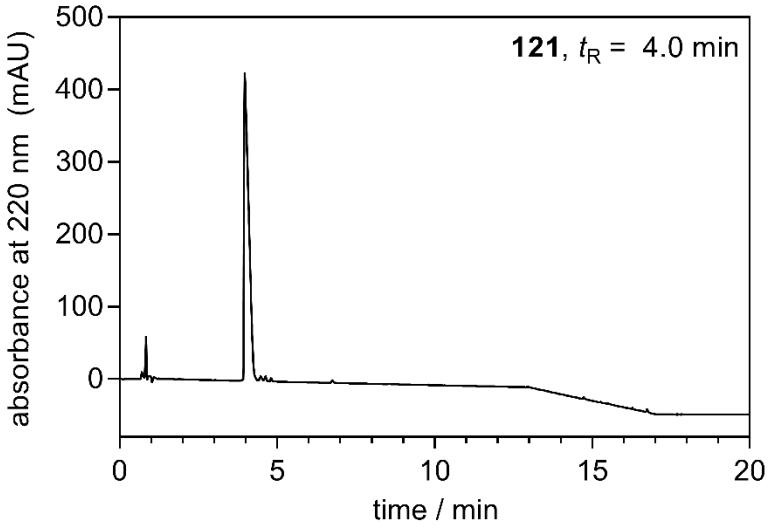
Chromatogram of the RP-HPLC analysis (purity control) of compound **118**



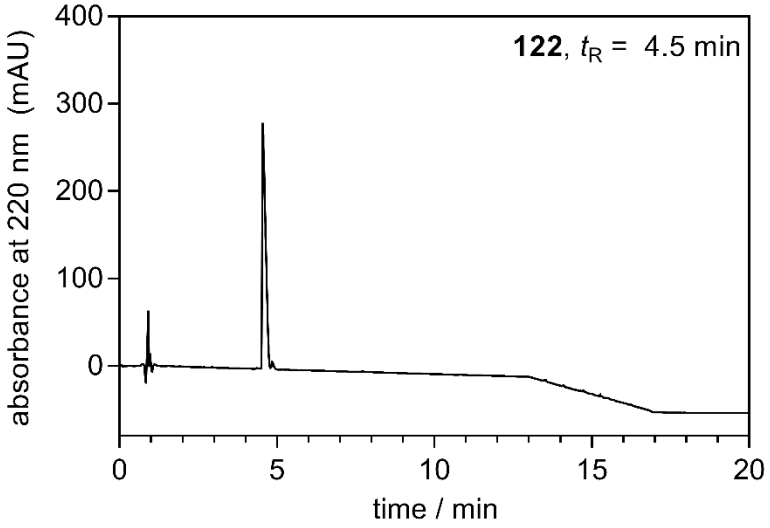
Chromatogram of the RP-HPLC analysis (purity control) of compound **119**



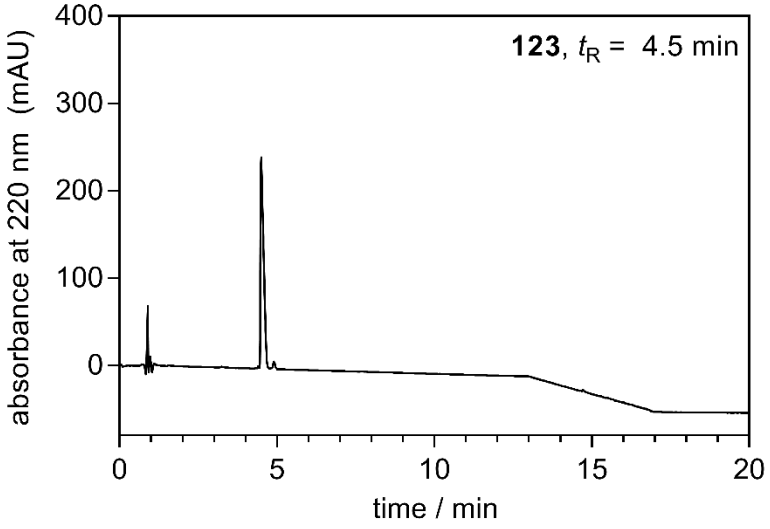
Chromatogram of the RP-HPLC analysis (purity control) of compound **120**



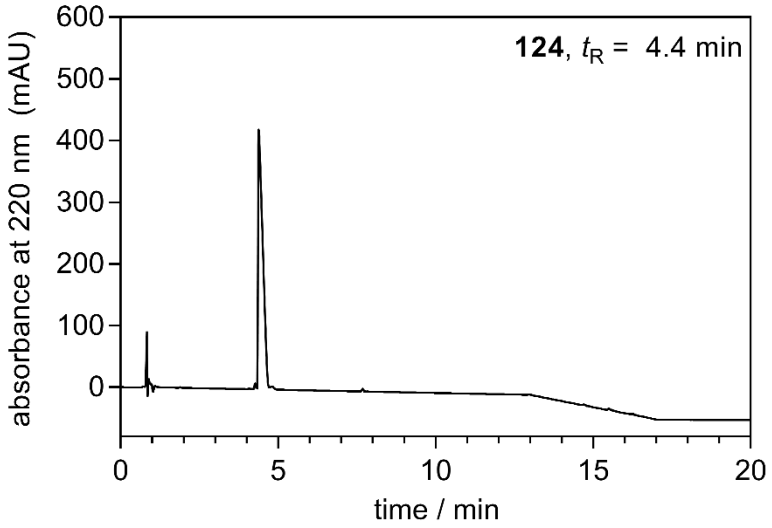
Chromatogram of the RP-HPLC analysis (purity control) of compound **121**



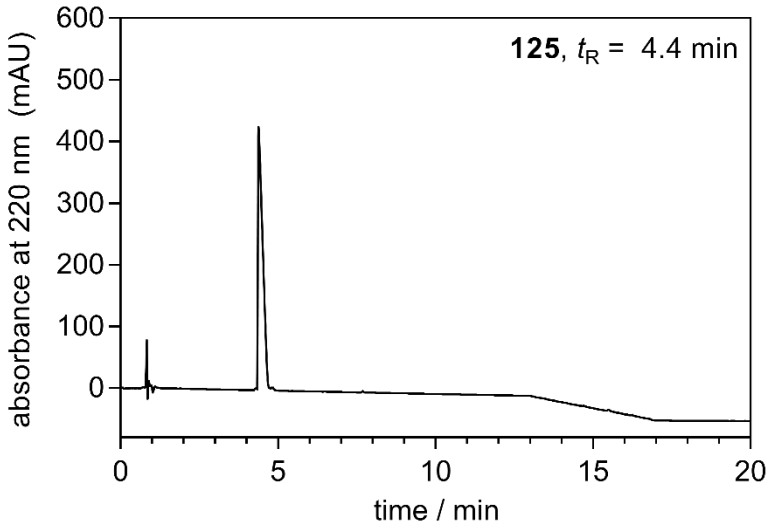
Chromatogram of the RP-HPLC analysis (purity control) of compound **122**



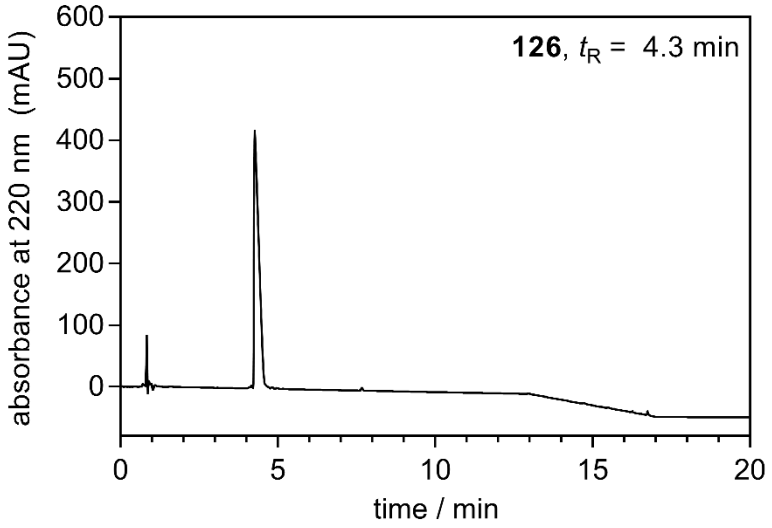
Chromatogram of the RP-HPLC analysis (purity control) of compound **123**



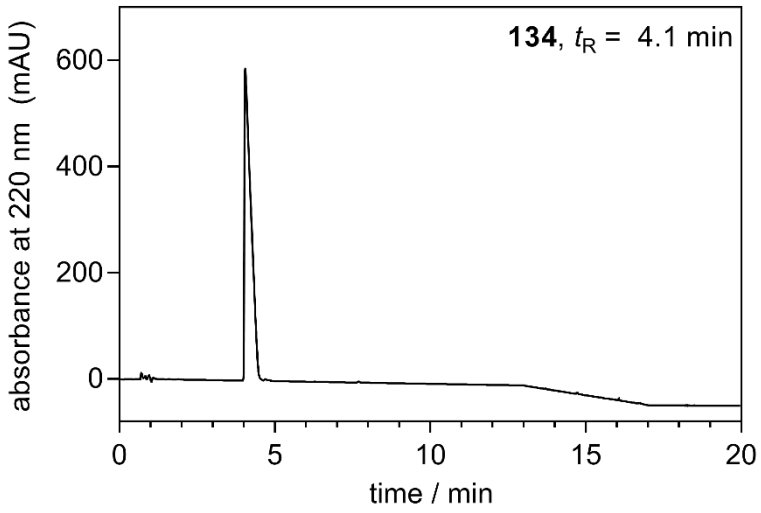
Chromatogram of the RP-HPLC analysis (purity control) of compound **124**



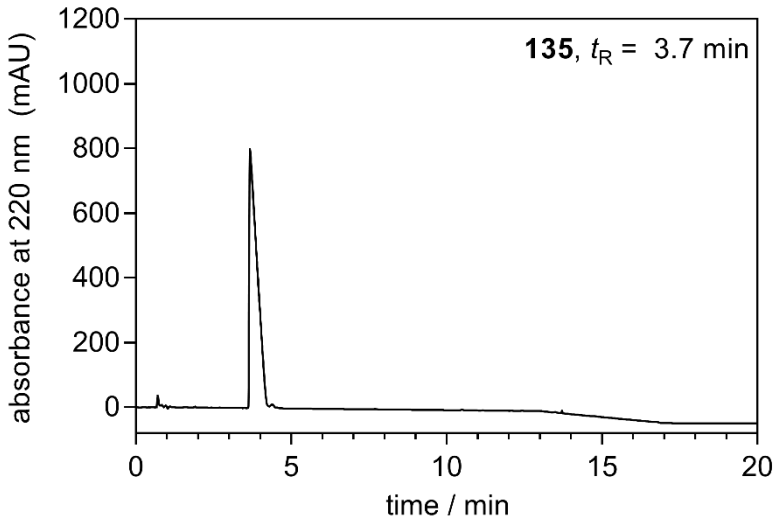
Chromatogram of the RP-HPLC analysis (purity control) of compound **125**



Chromatogram of the RP-HPLC analysis (purity control) of compound **126**

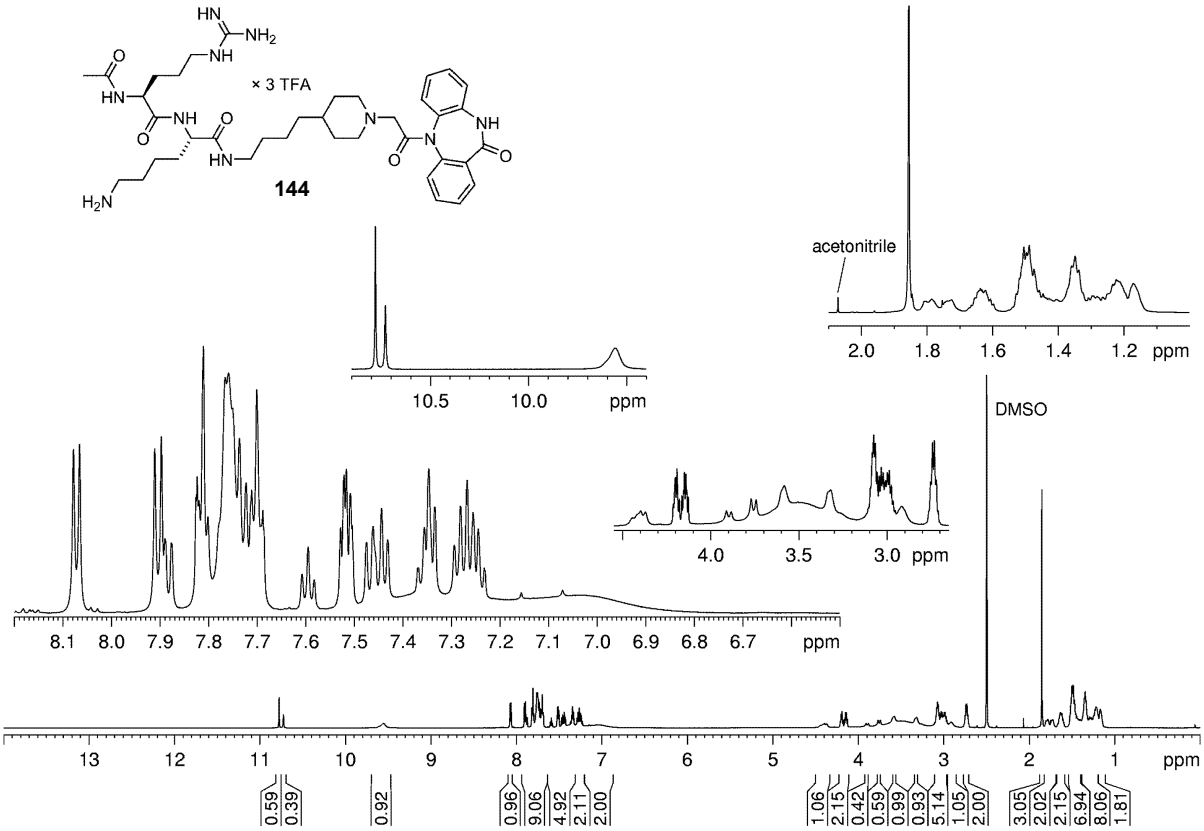


Chromatogram of the RP-HPLC analysis (purity control) of compound **134**

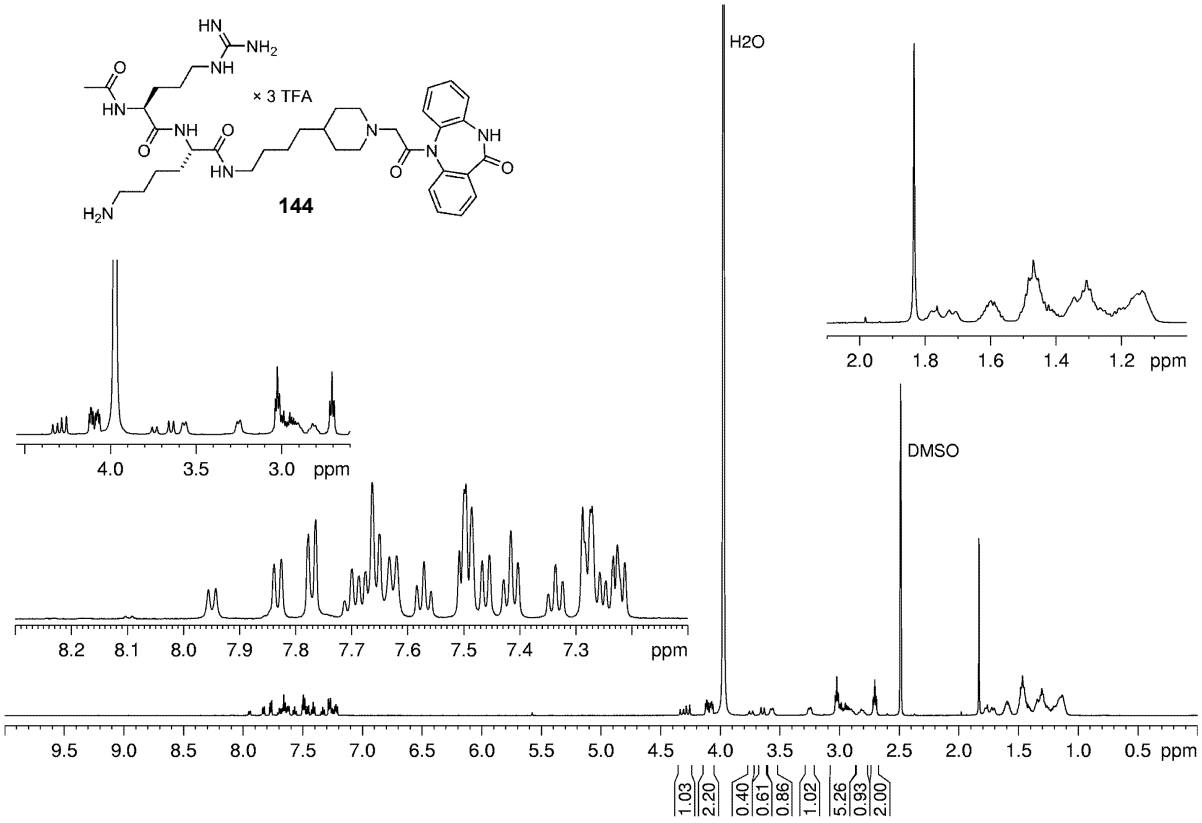


Chromatogram of RP-HPLC analysis (purity control) of compound **135**

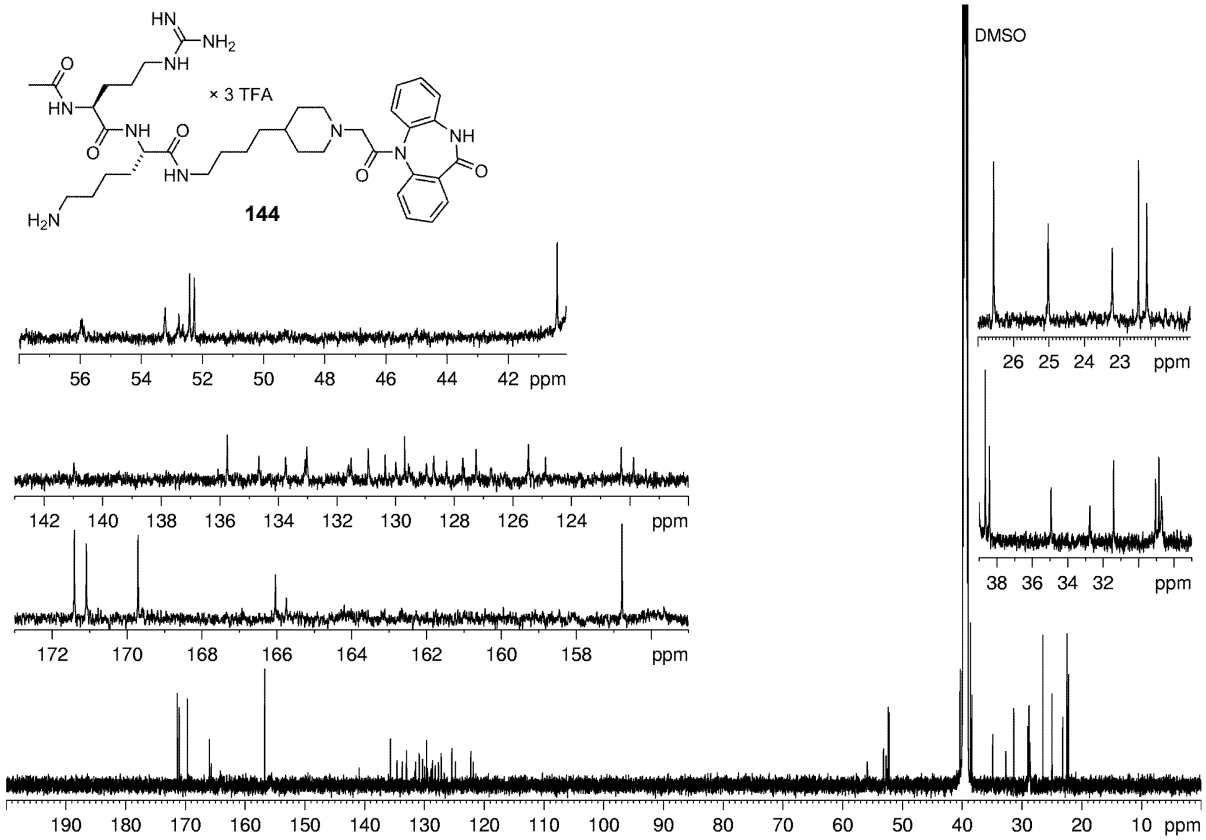
^{1}H and ^{13}C-NMR spectra of compounds 144-149 (Chapter 3)



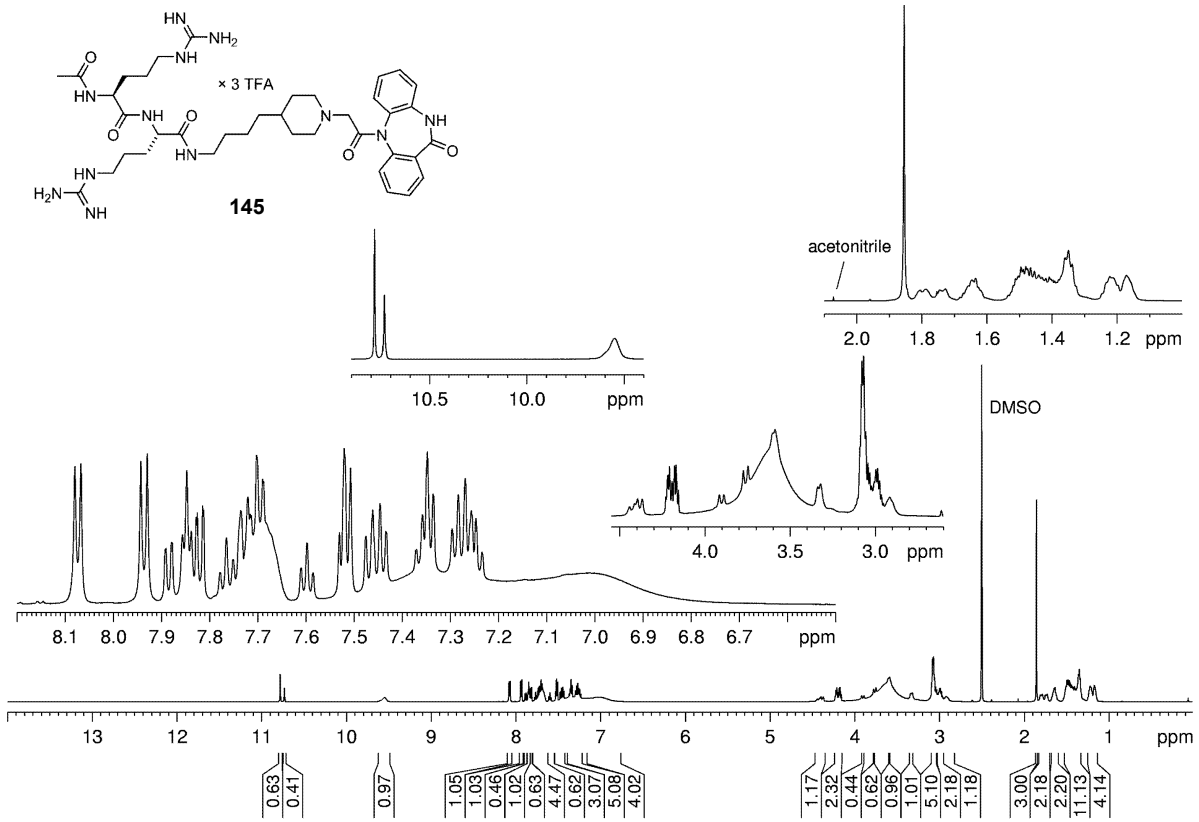
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **144**



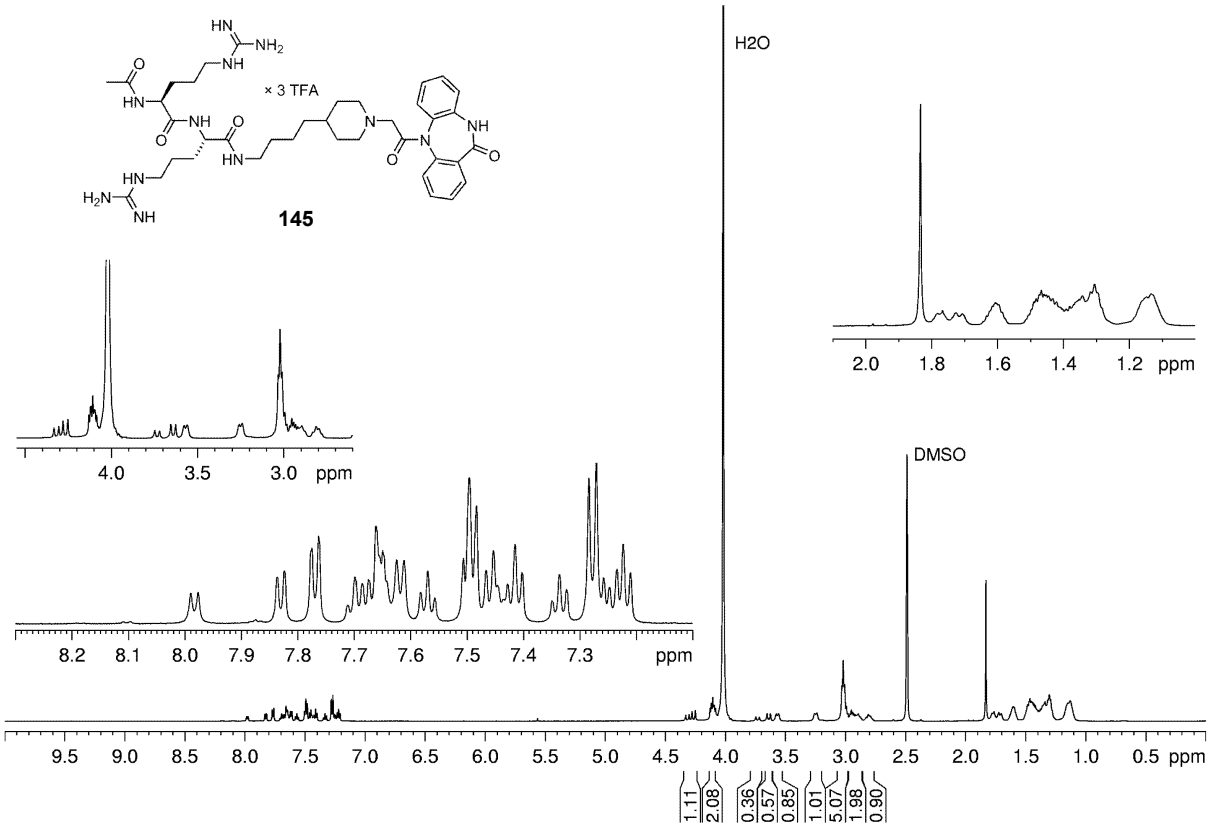
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **144**



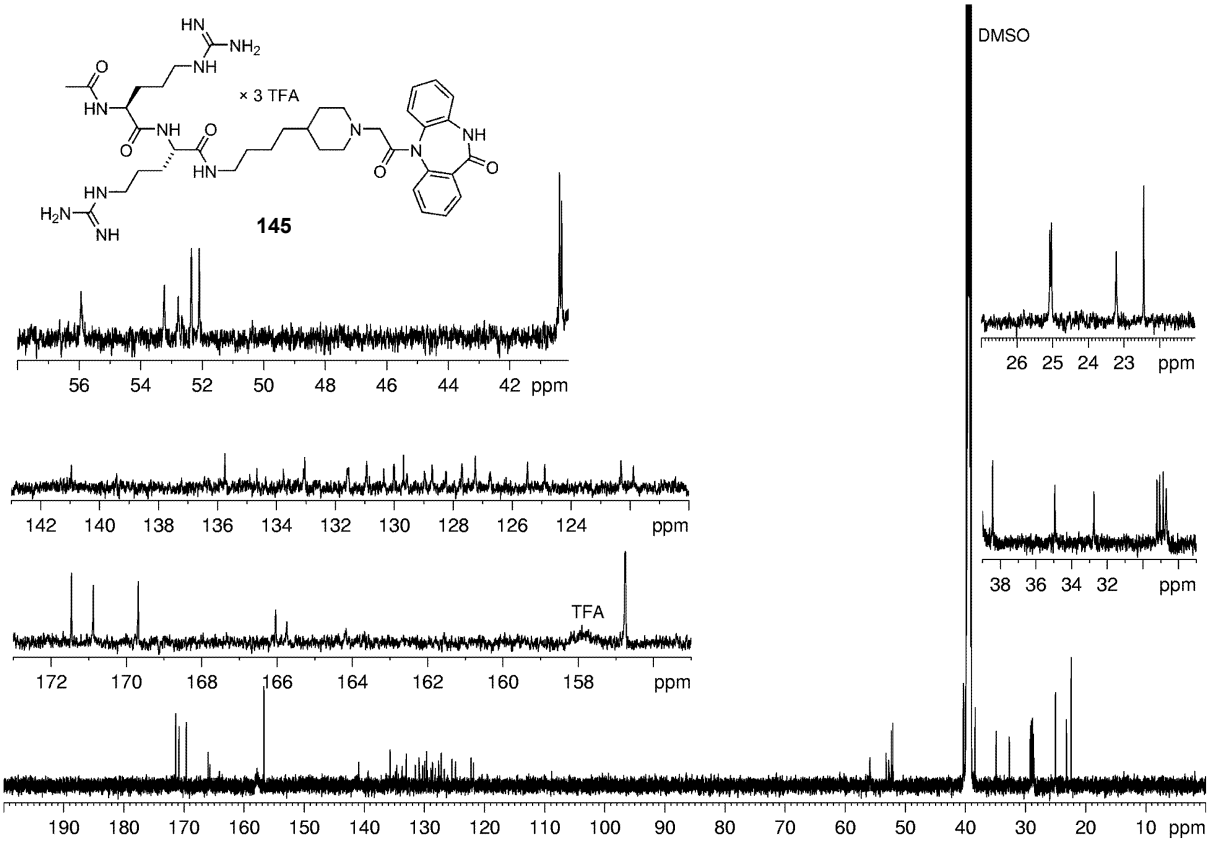
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **144**



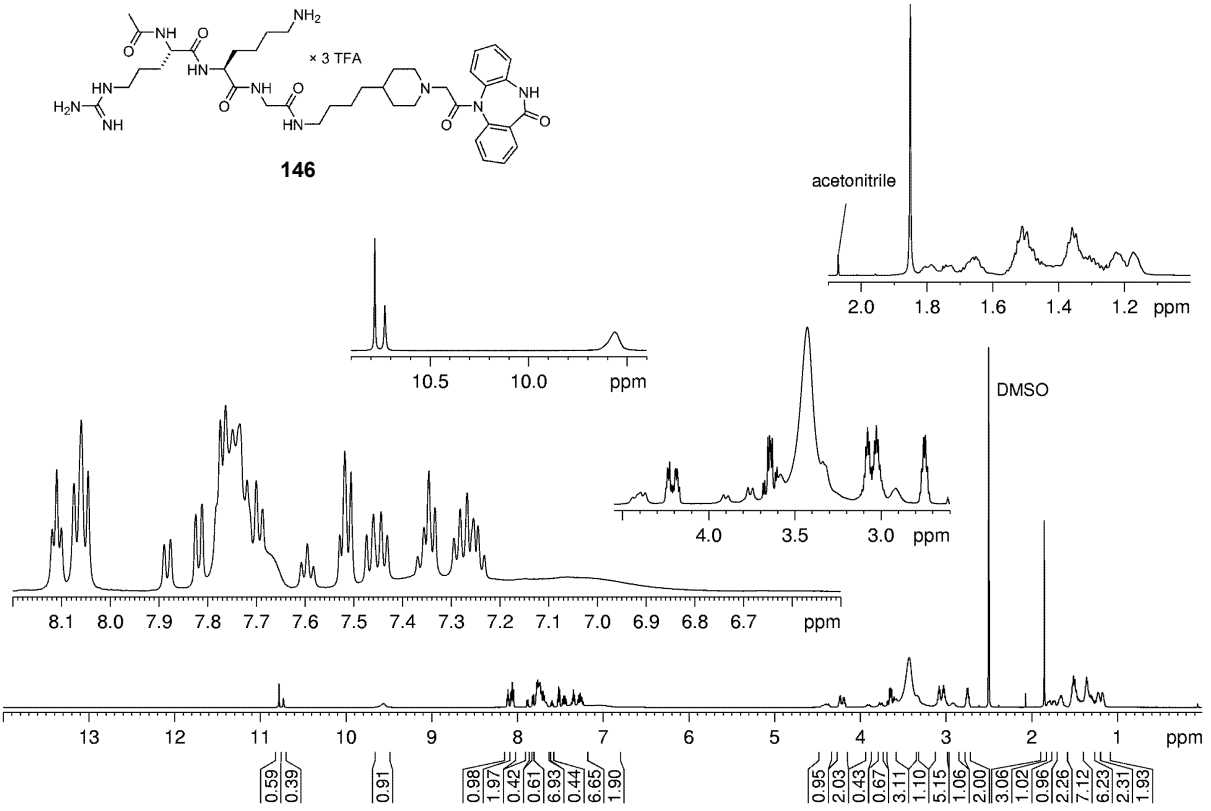
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **145**



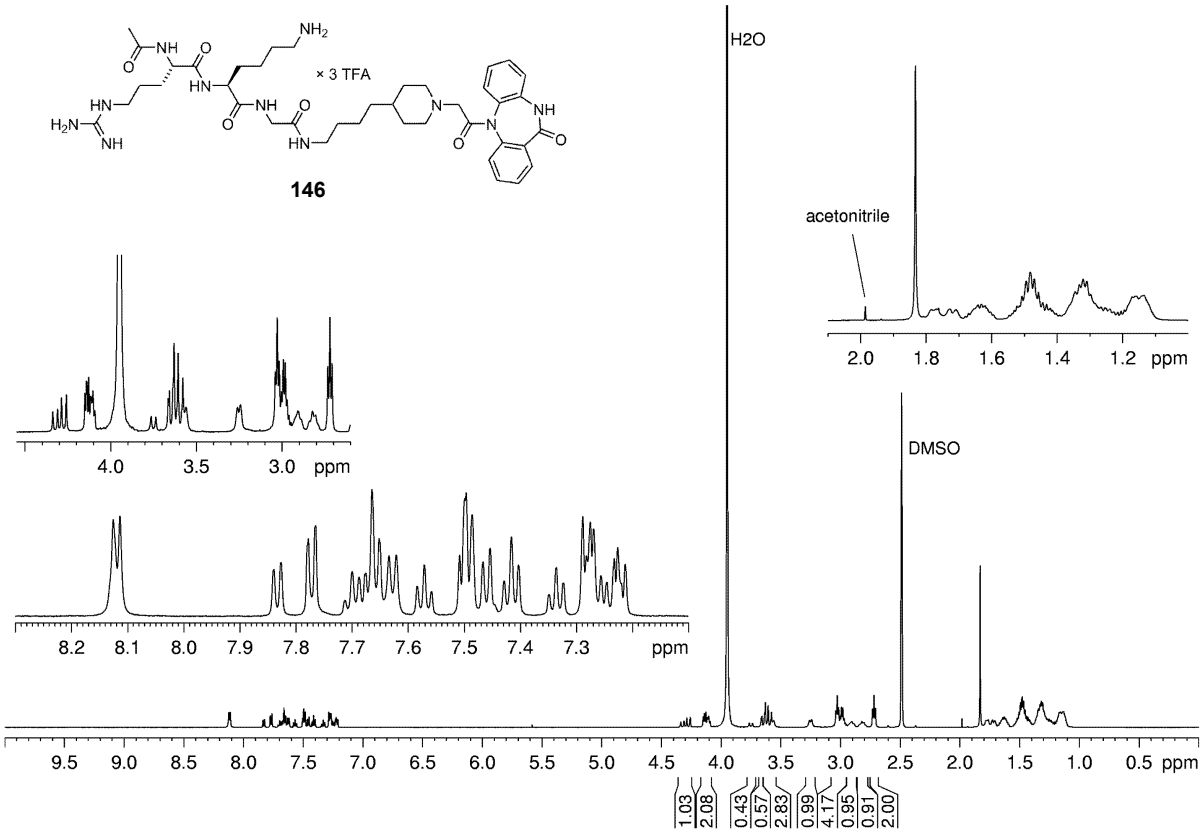
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **145**



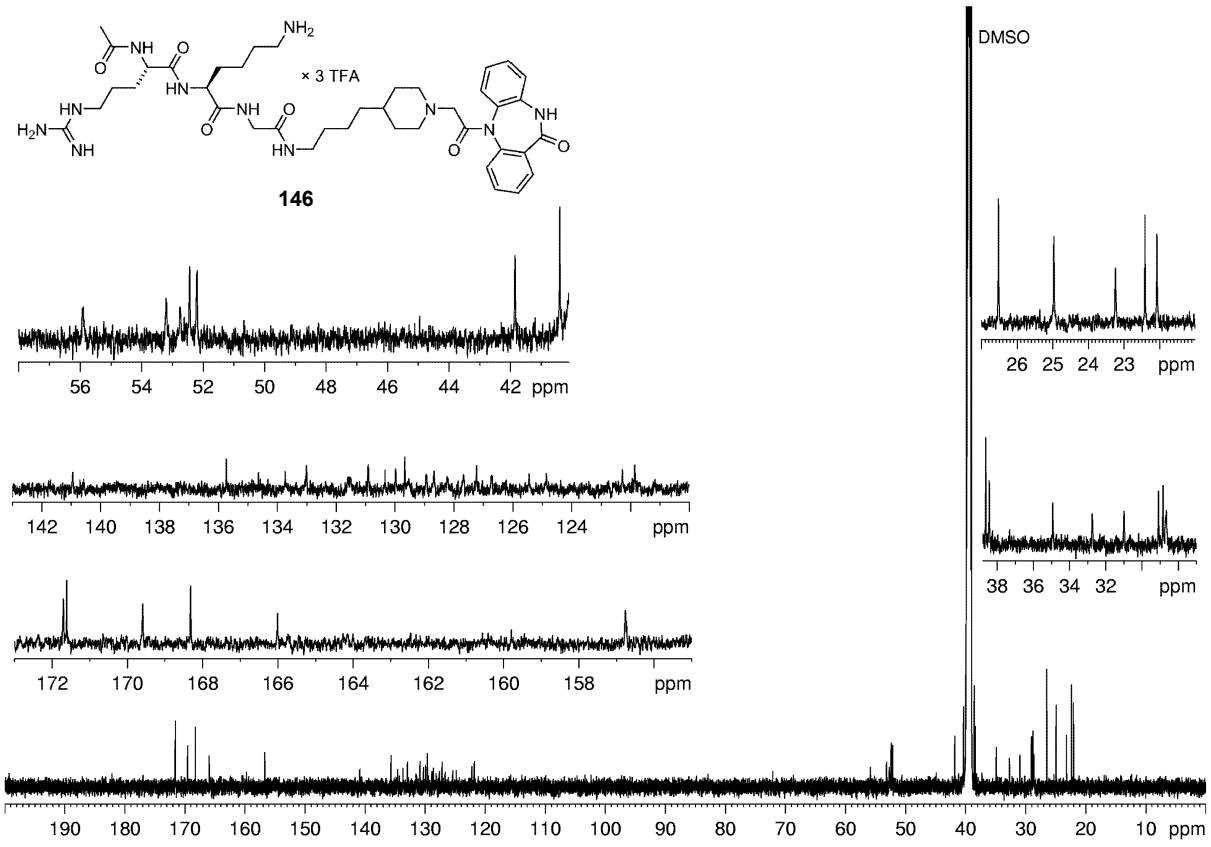
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **145**



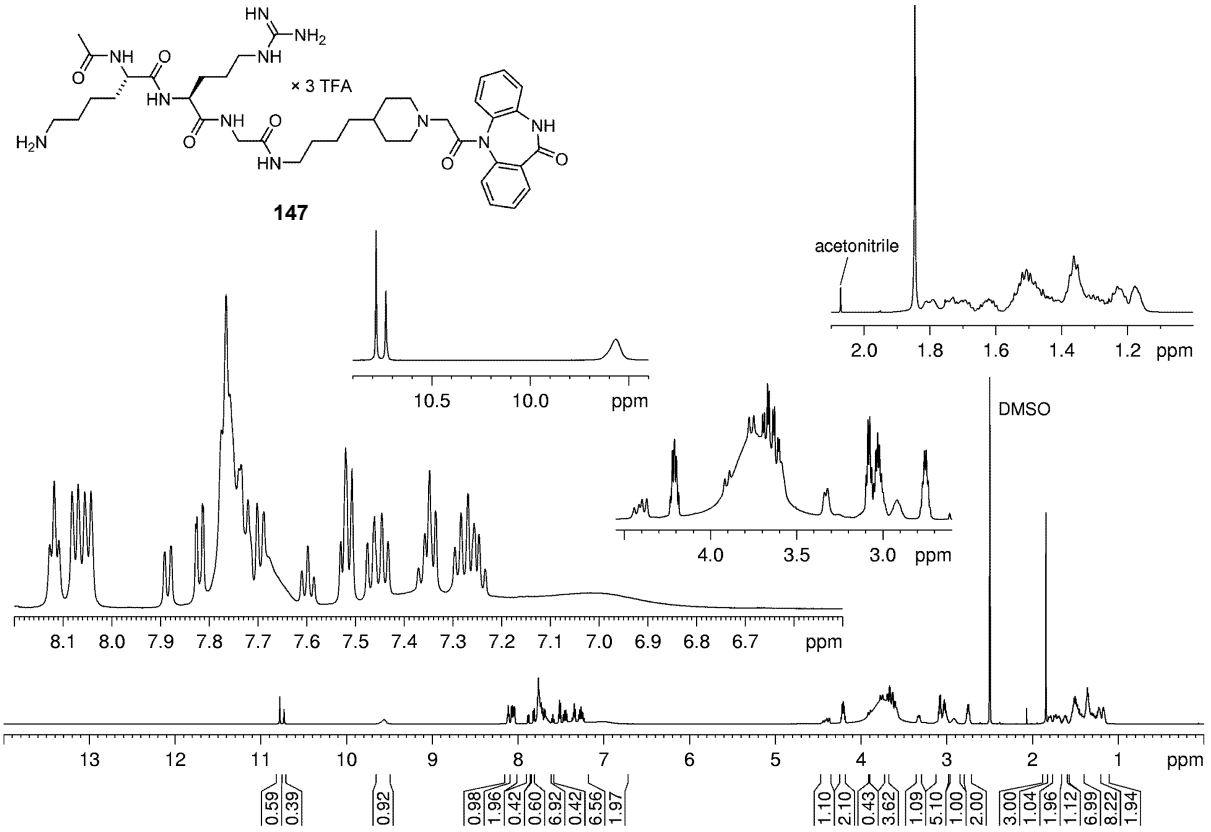
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **146**



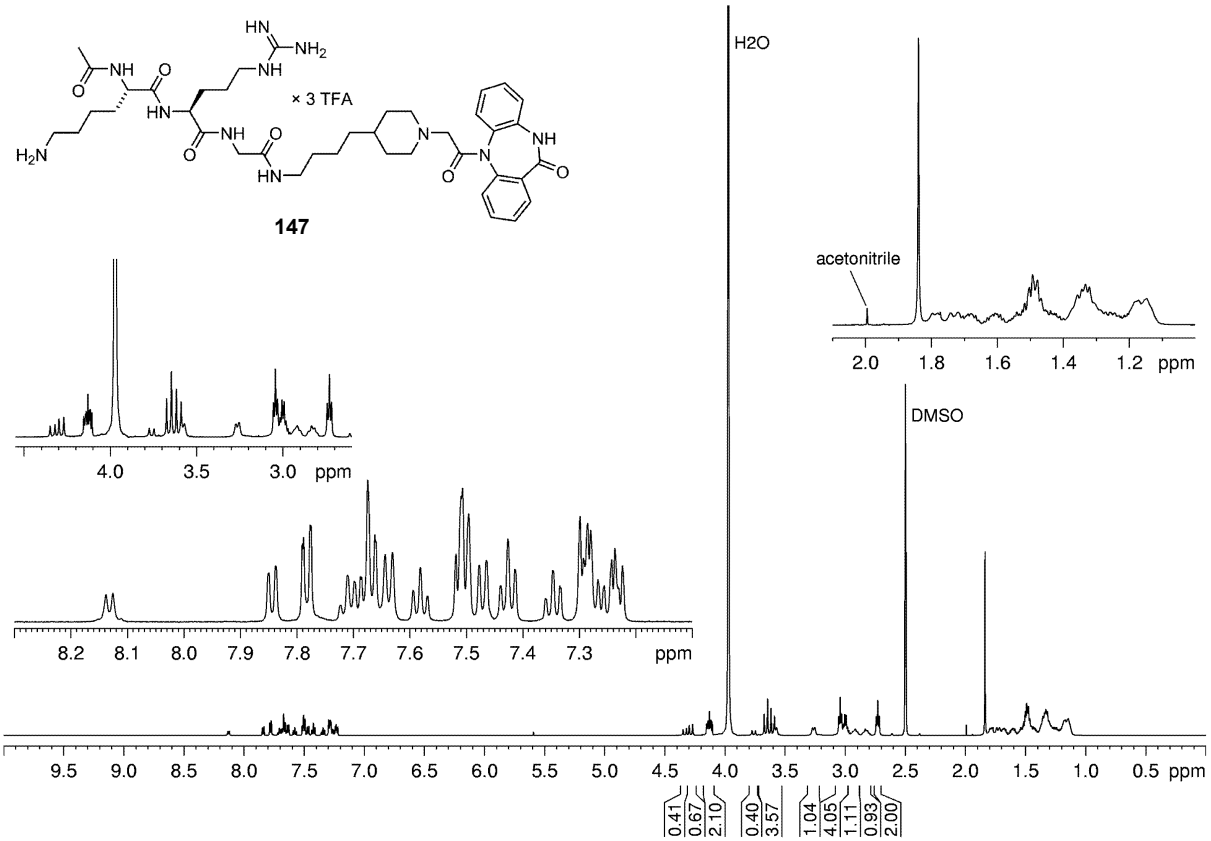
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **146**



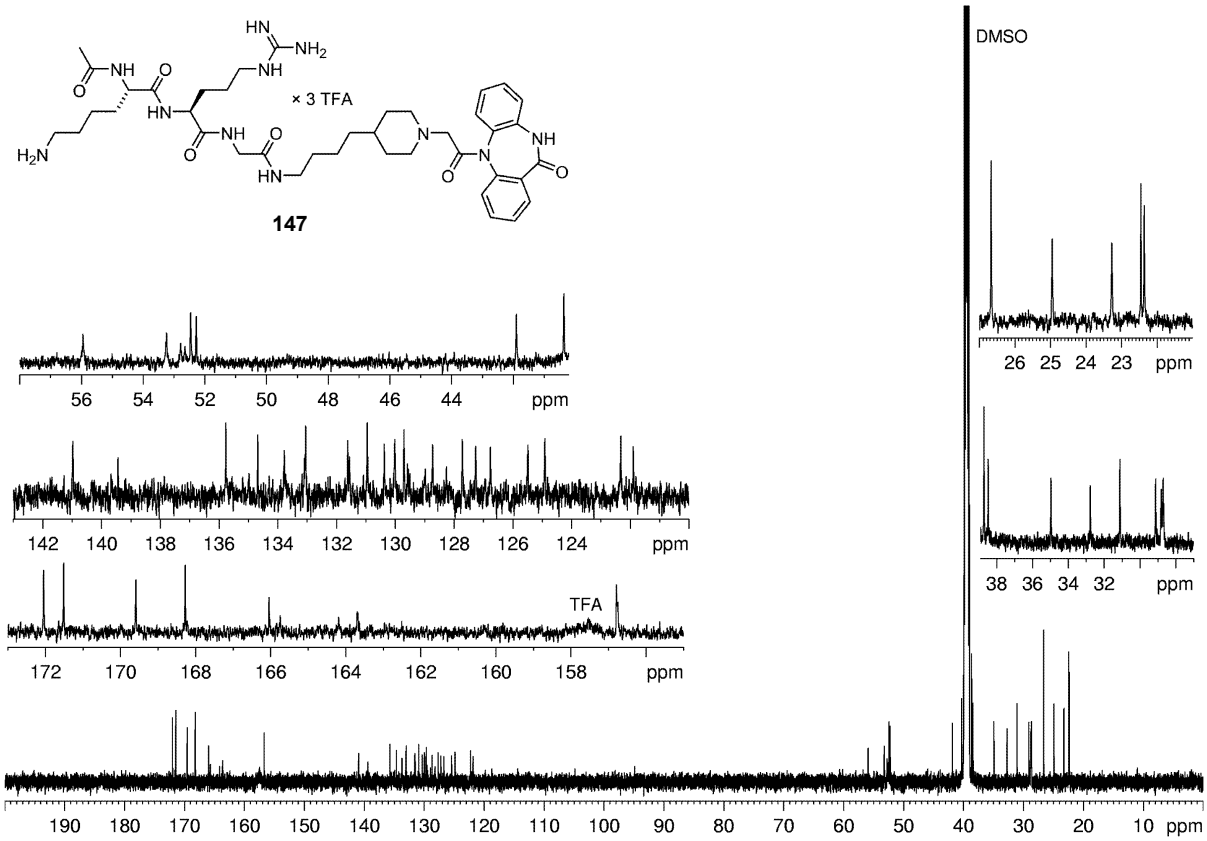
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **146**



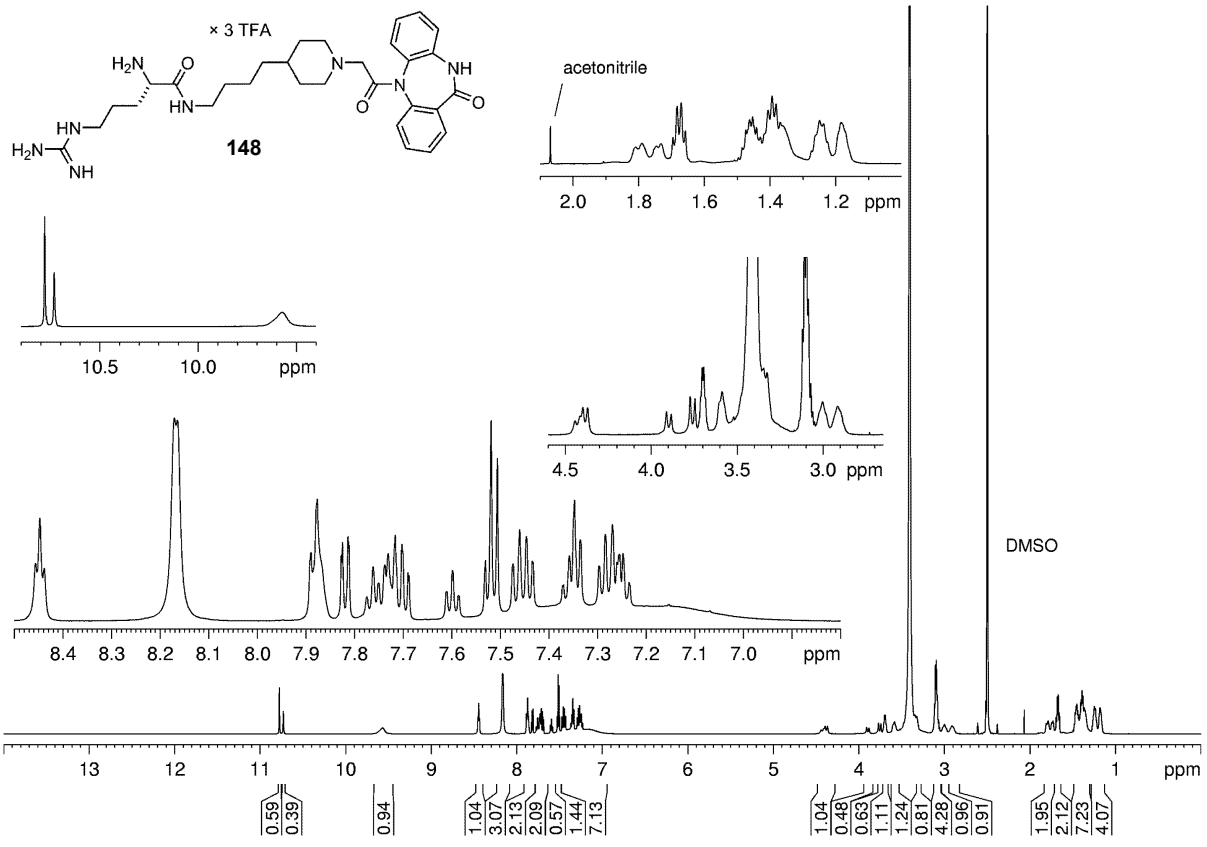
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **147**



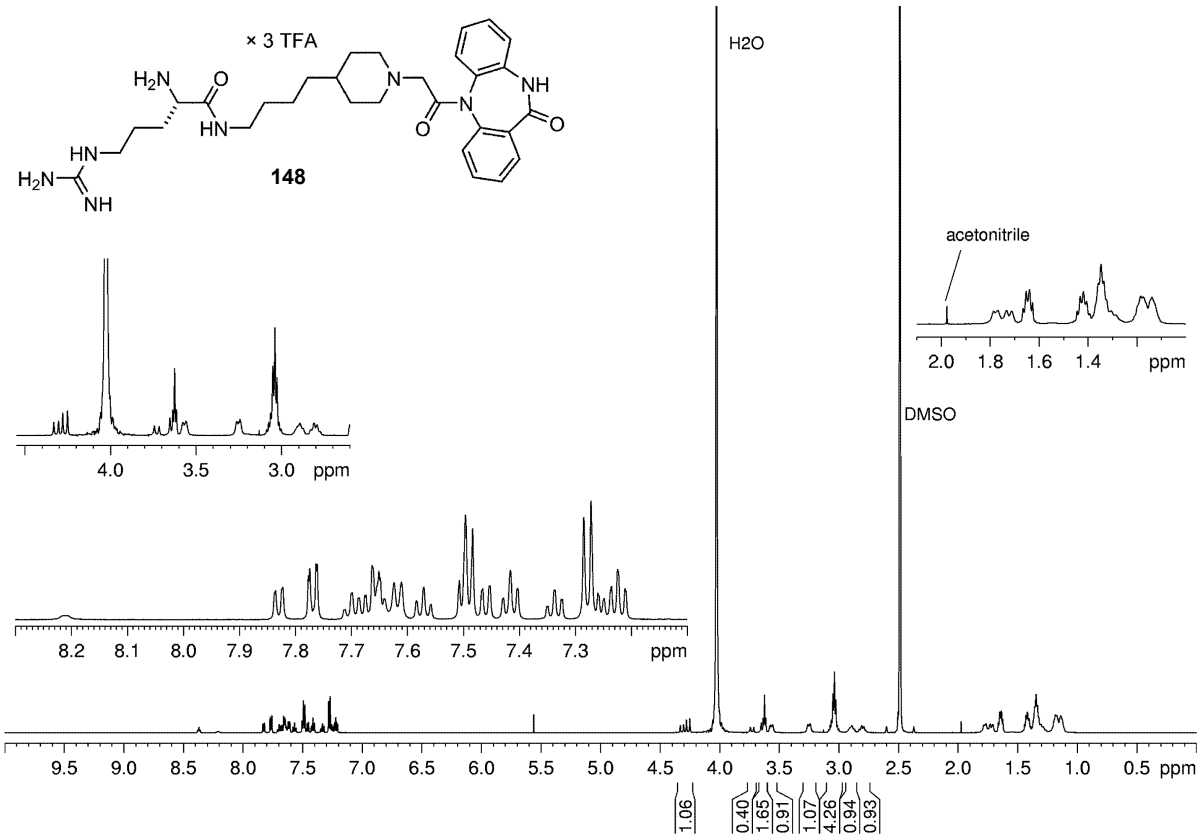
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **147**



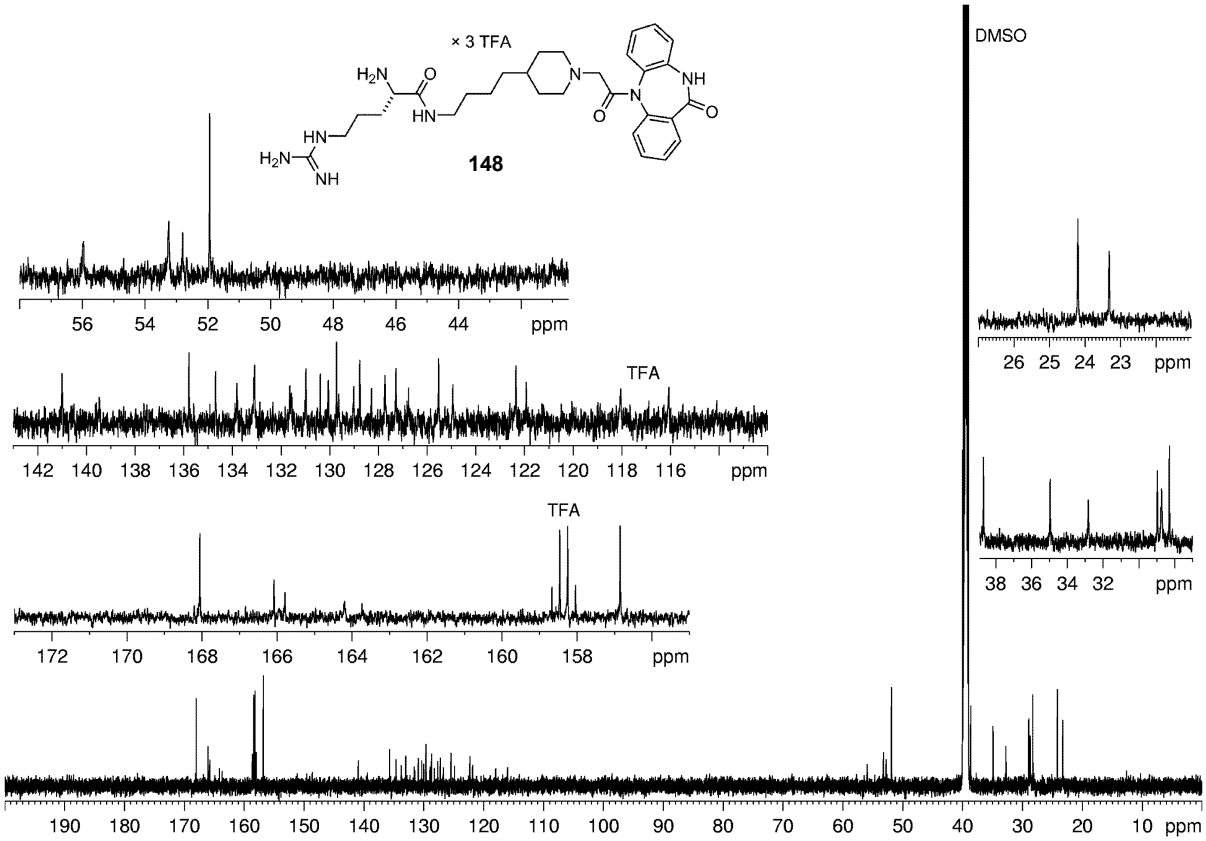
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **147**



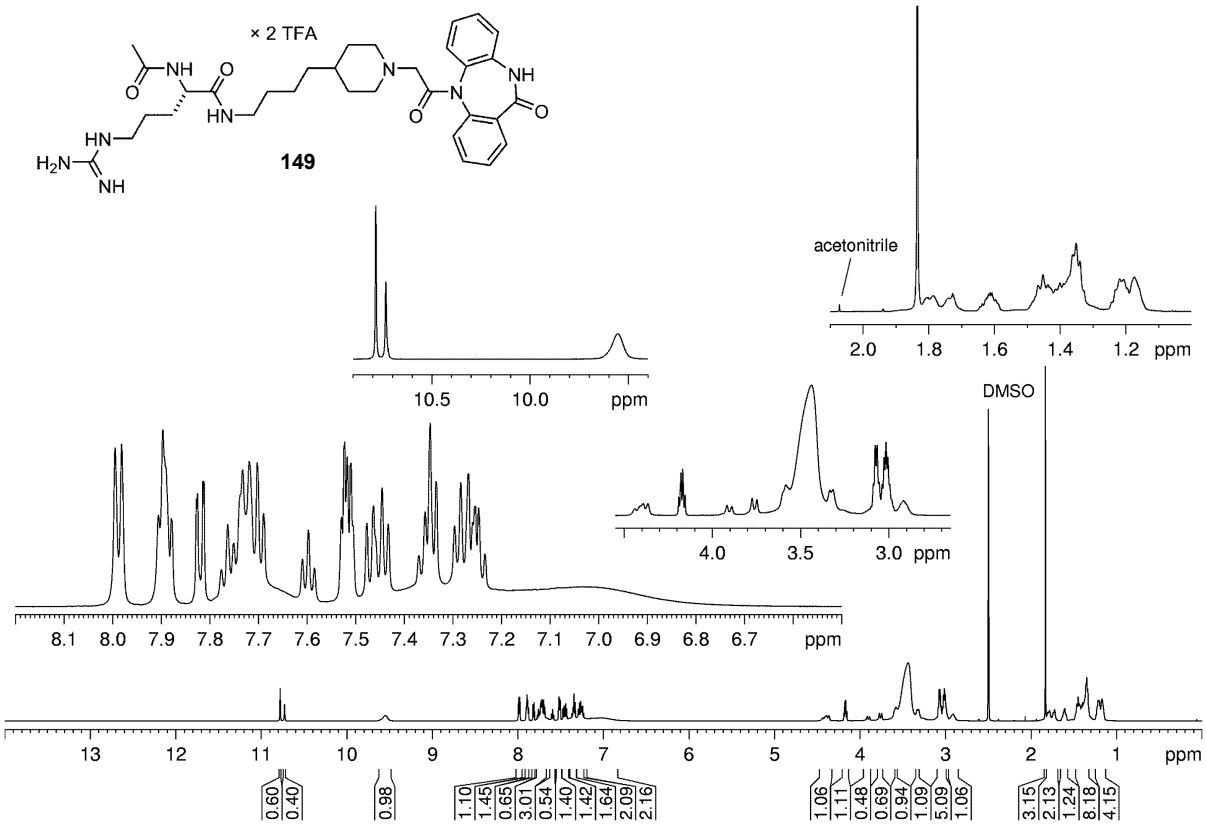
^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **148**



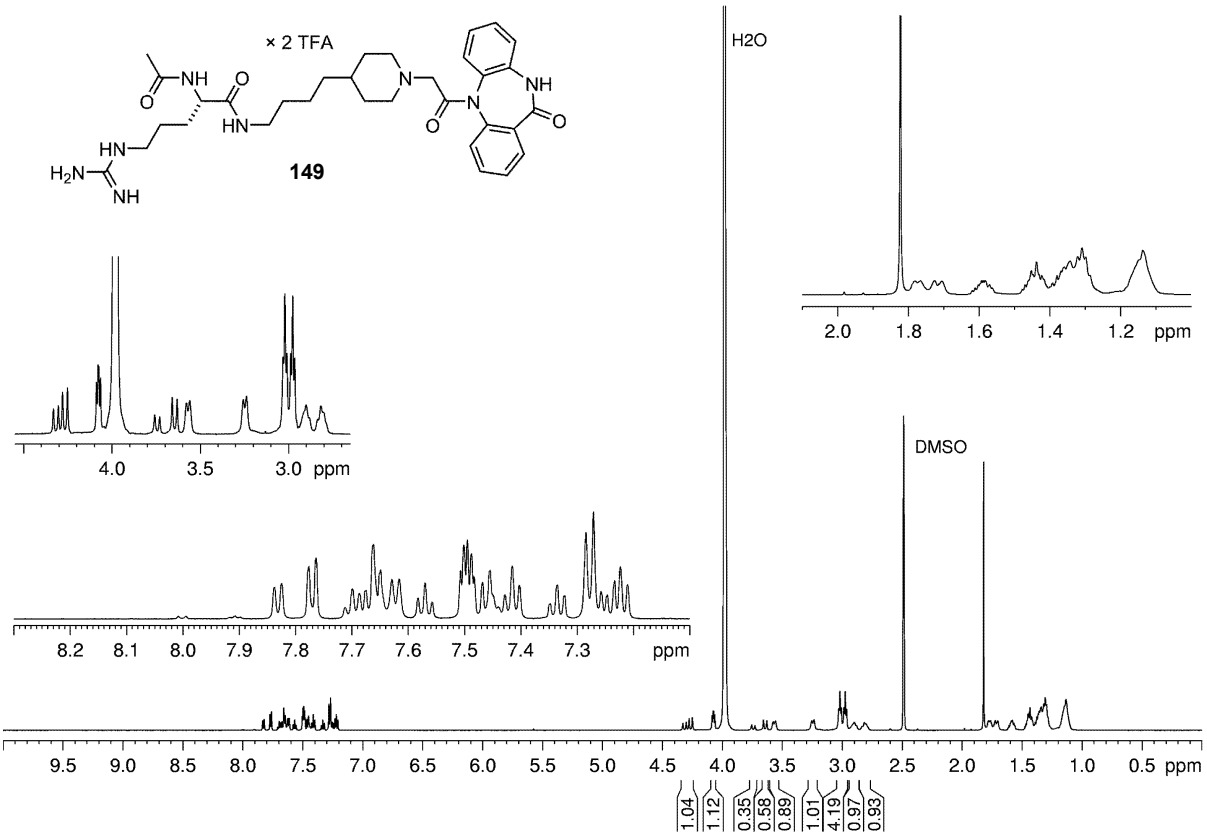
^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **148**



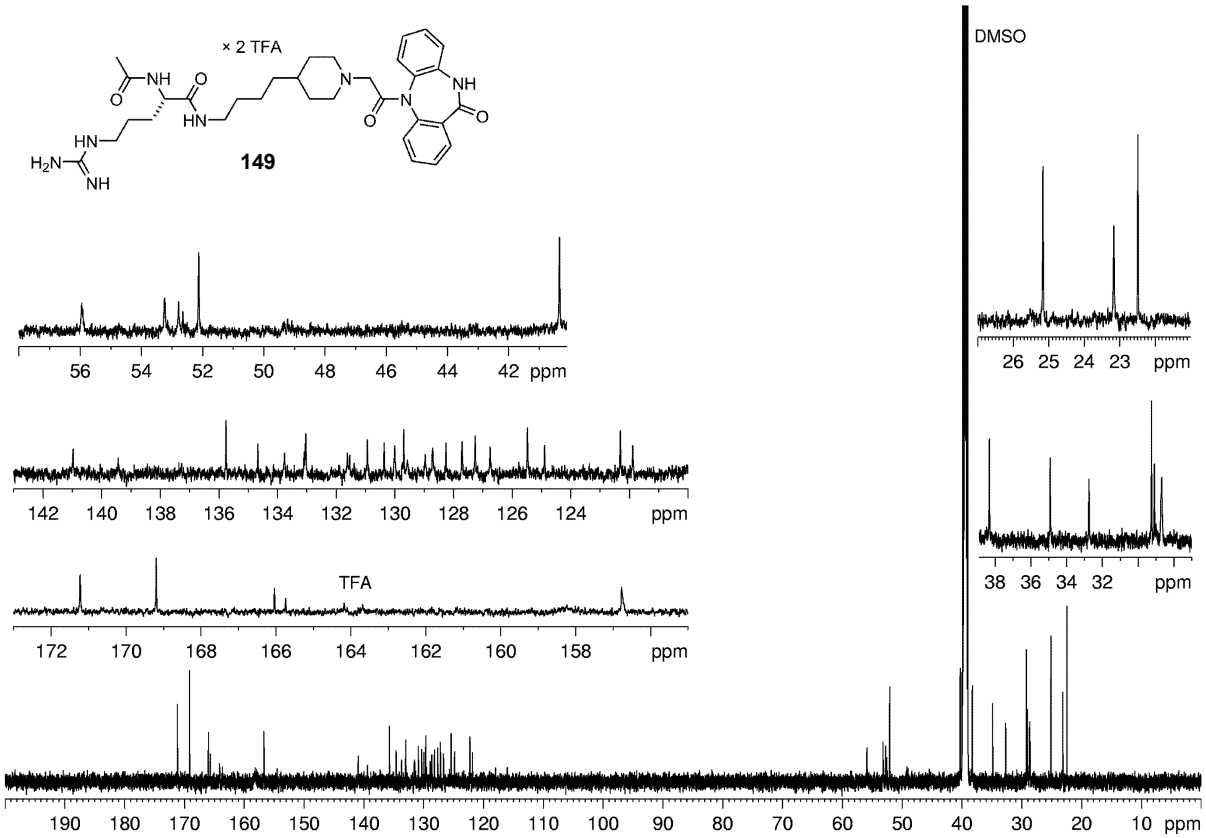
^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **148**



^{1}H-NMR spectrum (600 MHz, DMSO-d_6) of compound **149**

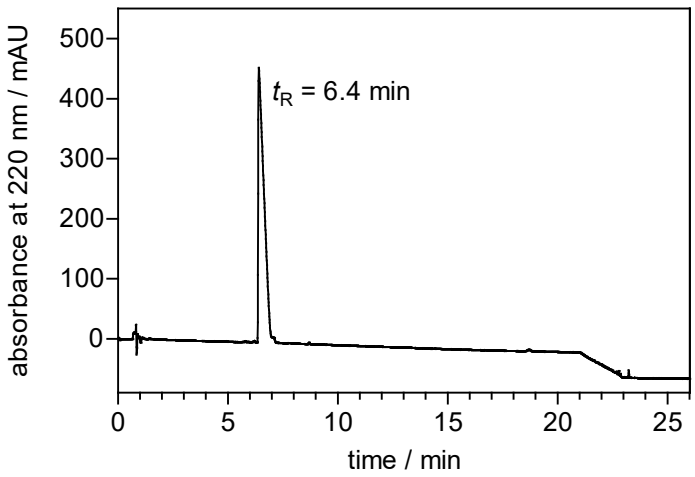


^{1}H-NMR spectrum (600 MHz, DMSO-d_6/D_2O 4:1 v/v) of compound **149**

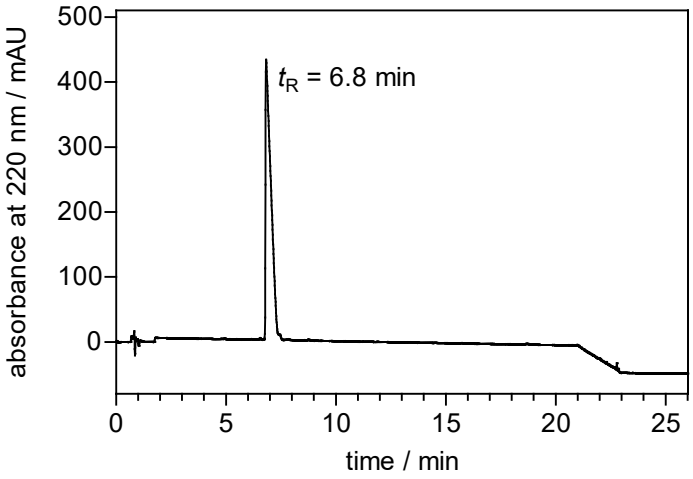


^{13}C-NMR spectrum (150 MHz, DMSO-d_6) of compound **149**

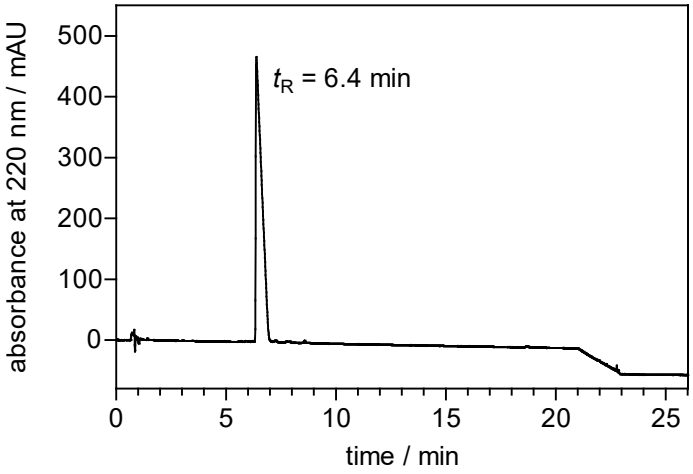
RP-HPLC chromatograms of compounds 144-149 (Chapter 3)



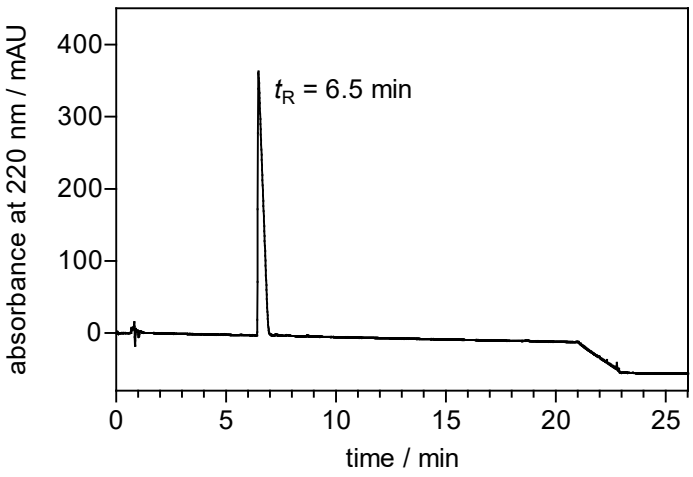
RP-HPLC analysis (purity control) of compound **144**



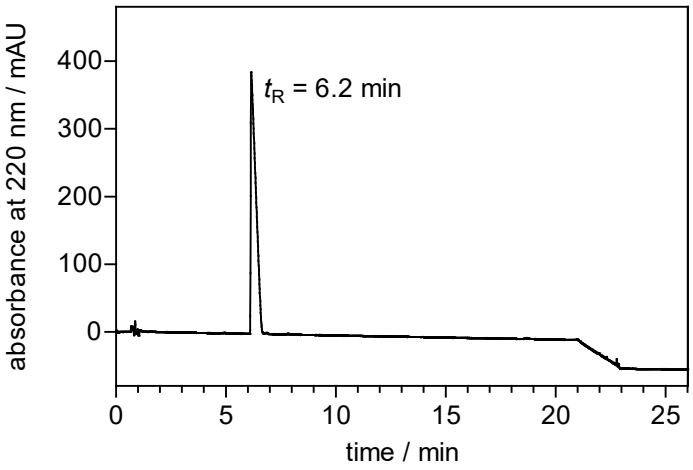
RP-HPLC analysis (purity control) of compound **145**



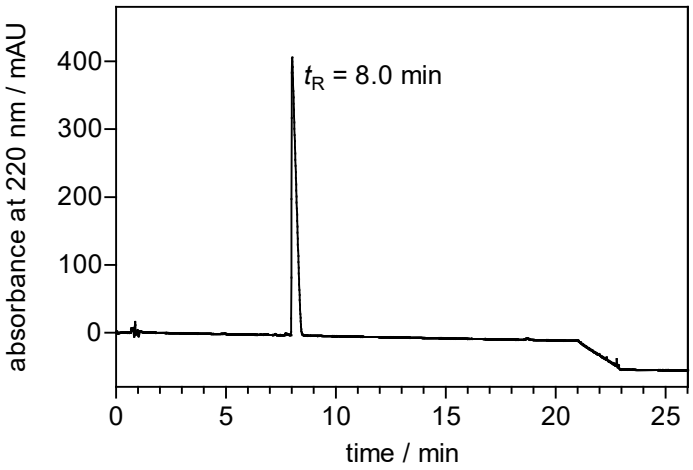
RP-HPLC analysis (purity control) of compound **146**



RP-HPLC analysis (purity control) of compound **147**

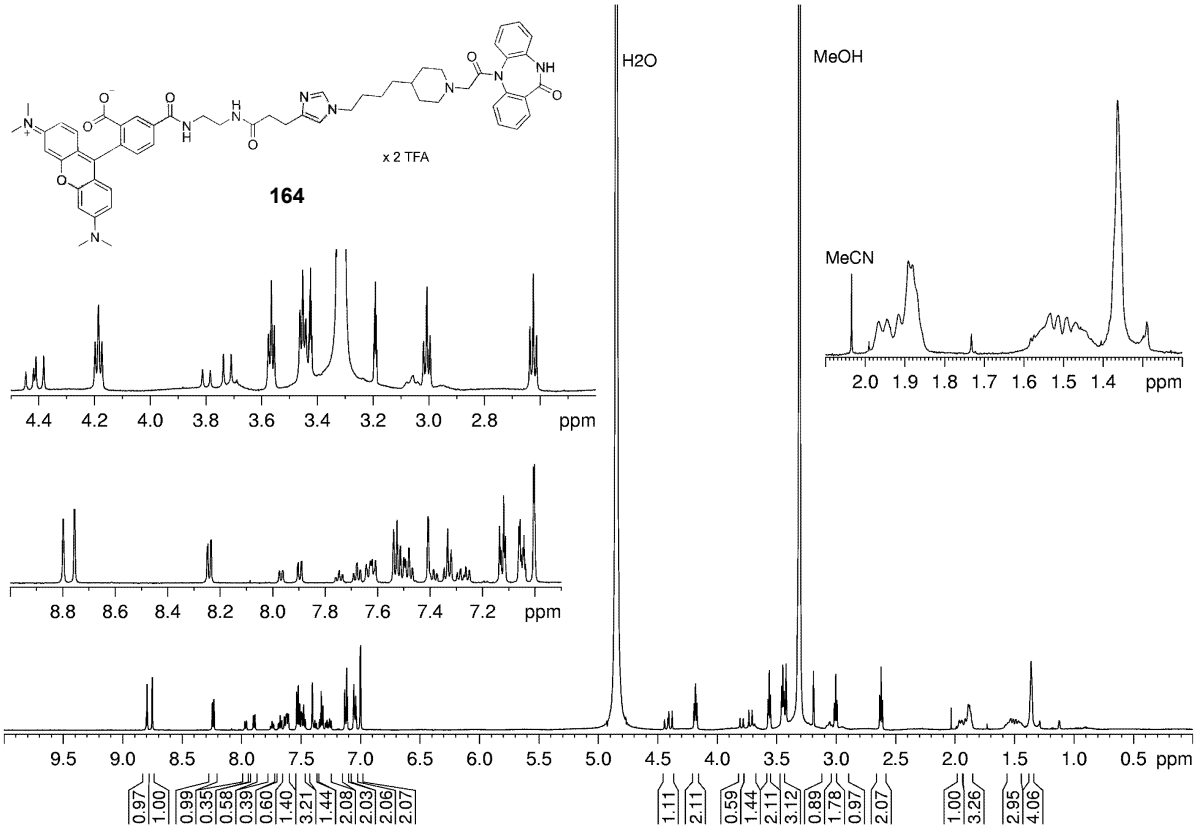


RP-HPLC analysis (purity control) of compound **148**

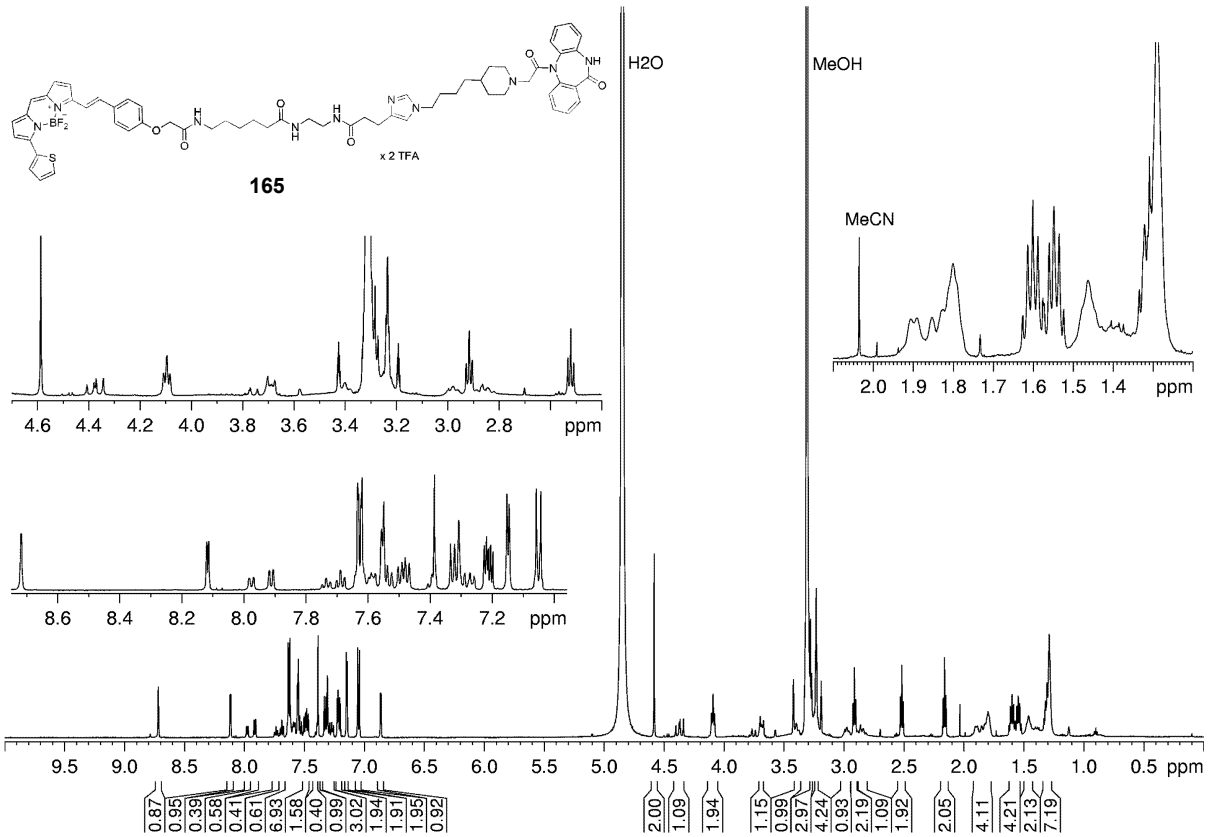


RP-HPLC analysis (purity control) of compound **149**

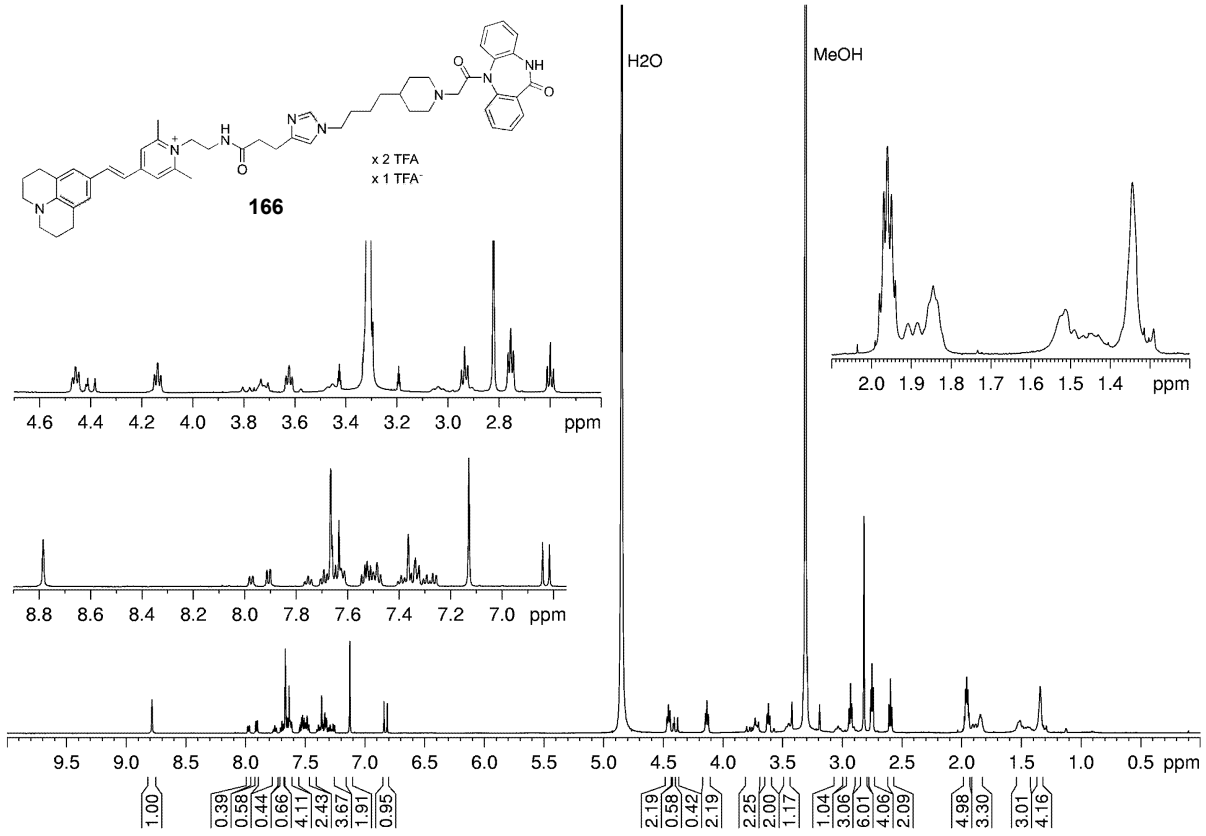
^{1}H and spectra of compounds 164-169 (Chapter 4)



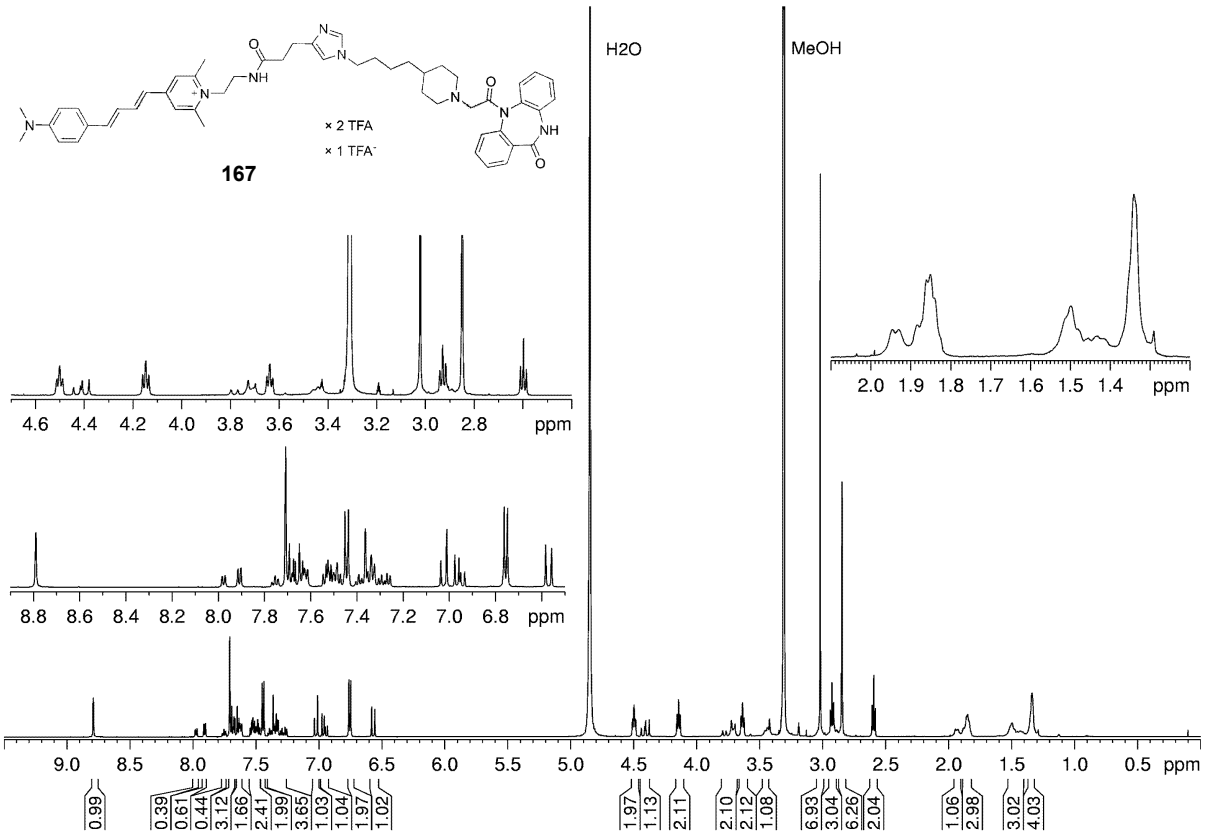
^{1}H-NMR spectrum (600 MHz, MeOH-d_4) of compound **164**



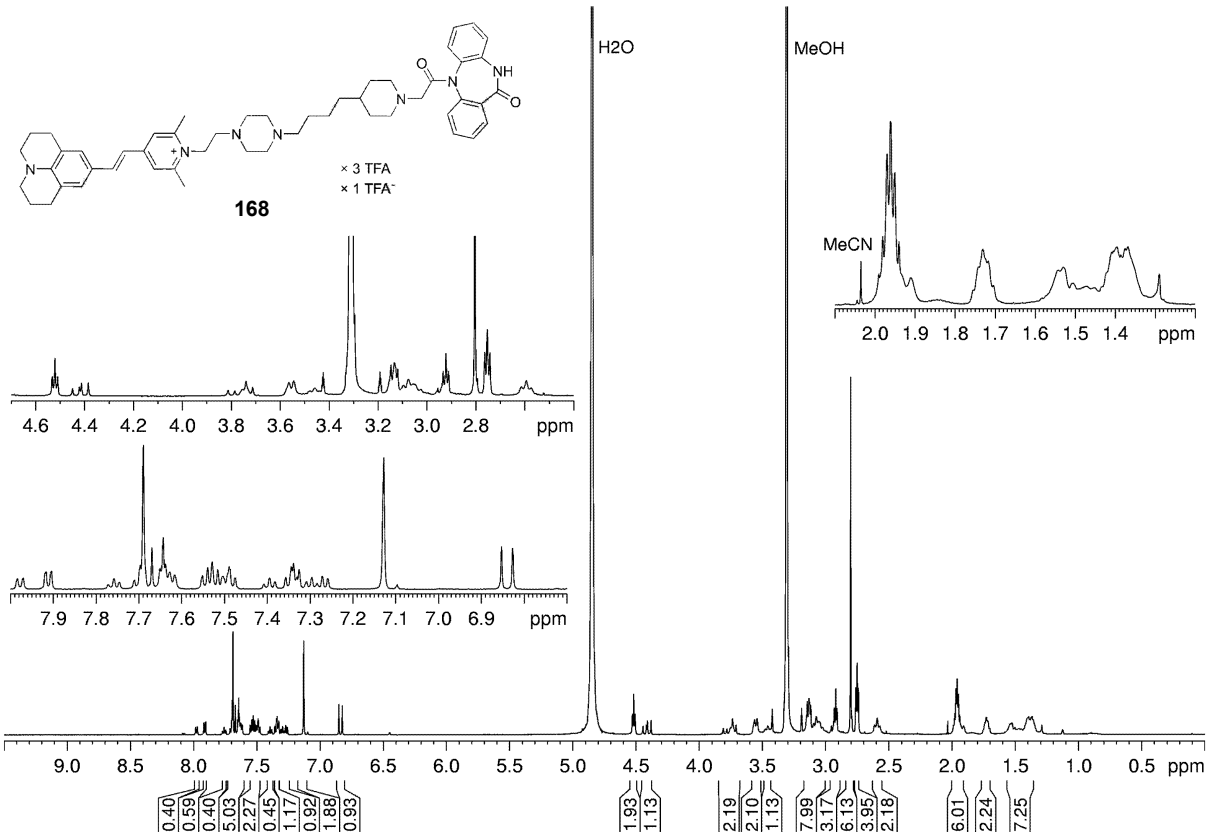
^{1}H-NMR spectrum (600 MHz, MeOH-d_4) of compound **165**



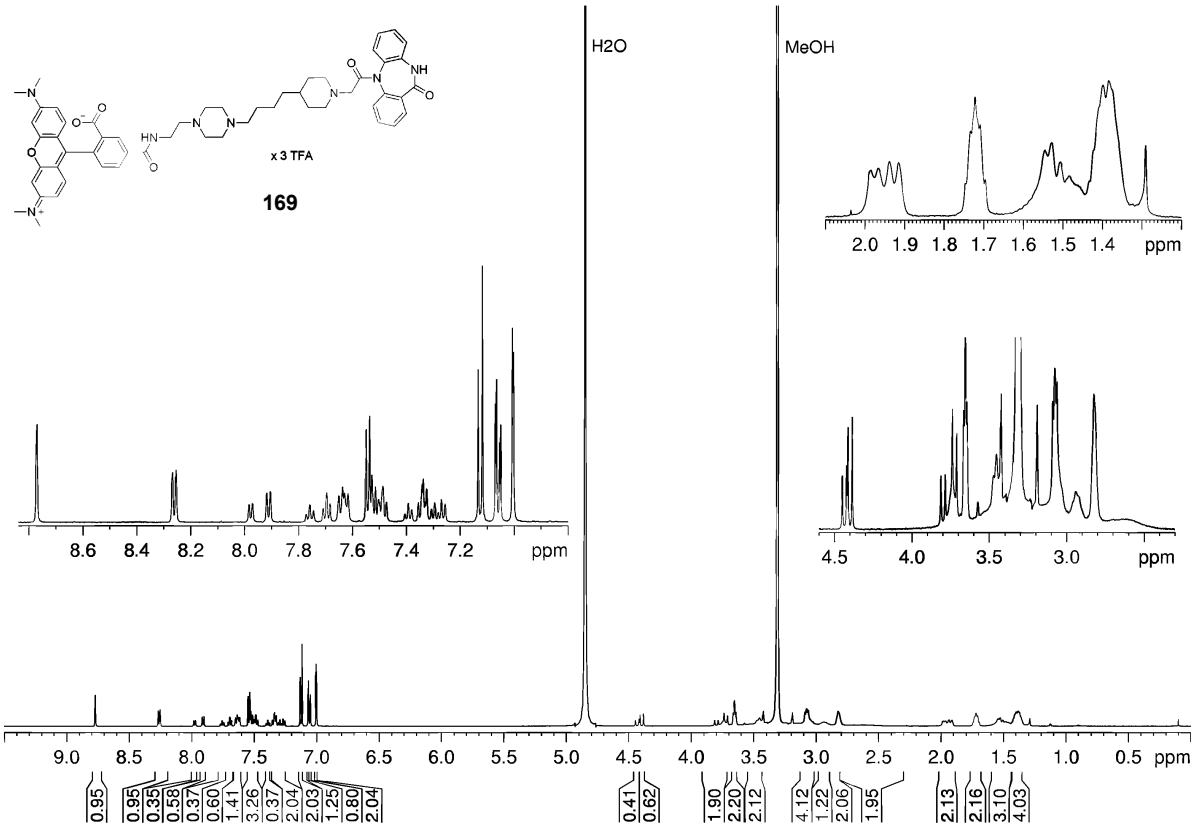
^{1}H-NMR spectrum (600 MHz, MeOH-d_4) of compound **166**



^{1}H-NMR spectrum (600 MHz, MeOH-d_4) of compound **167**

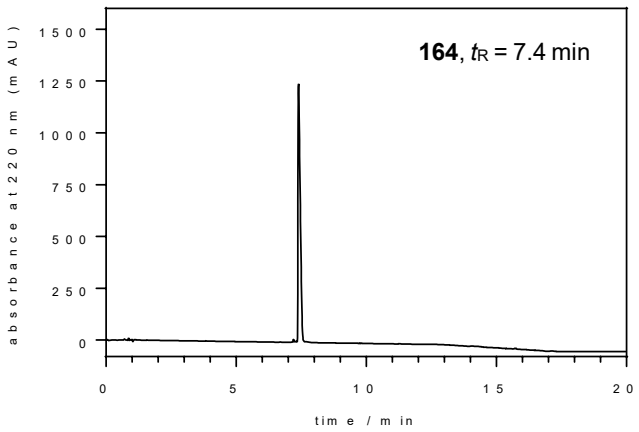


^{1}H-NMR spectrum (600 MHz, MeOH-d_4) of compound **168**

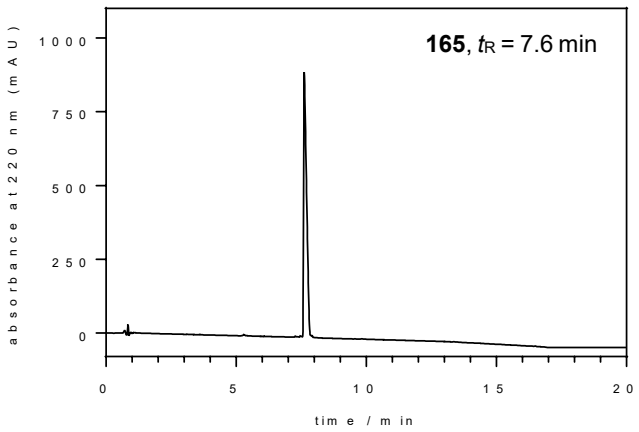


^{1}H-NMR spectrum (600 MHz, MeOH-d_4) of compound **169**

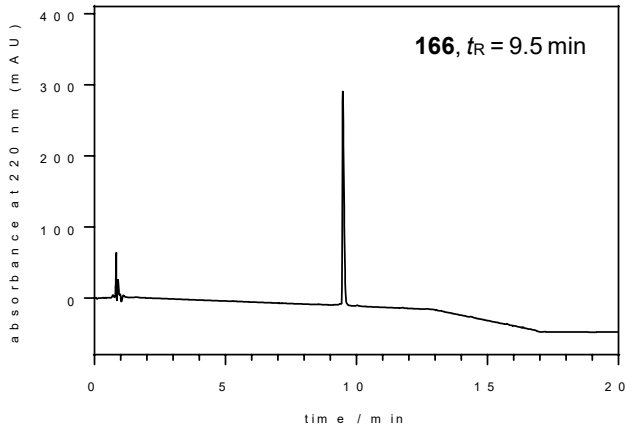
RP-HPLC chromatograms of compounds 164-169 (Chapter 4)



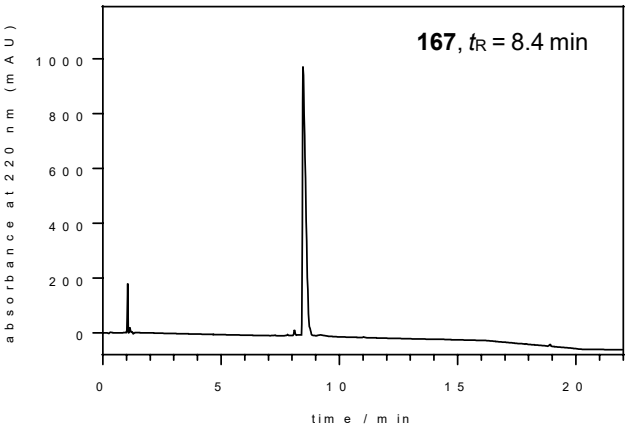
Chromatogram of the RP-HPLC analysis (purity control) of compound **164**



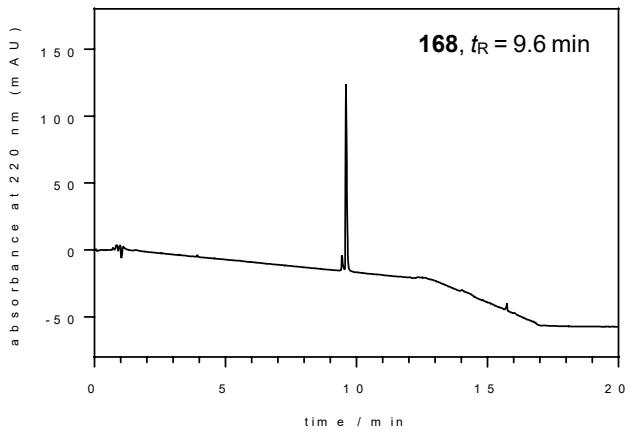
Chromatogram of the RP-HPLC analysis (purity control) of compound **165**



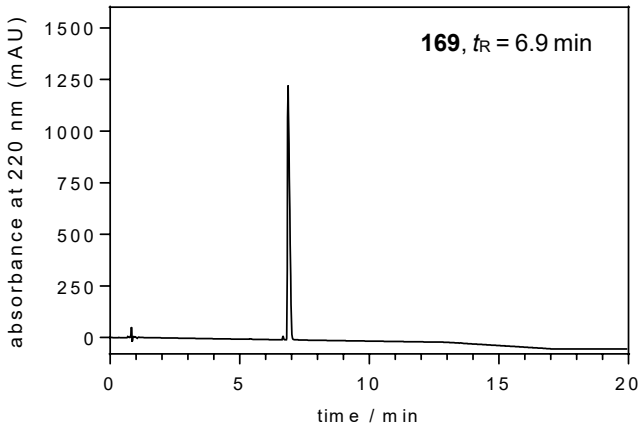
Chromatogram of the RP-HPLC analysis (purity control) of compound **166**



Chromatogram of the RP-HPLC analysis (purity control) of compound **167**



Chromatogram of the RP-HPLC analysis (purity control) of compound **168**



Chromatogram of the RP-HPLC analysis (purity control) of compound **169**

Abbreviations

Agb	shorter Arginine homologue with an ethylene instead of a trimethylene moiety in the side chain
aq.	aqueous
Boc	tert-butoxycarbonyl
br	broad signal
BSA	bovine serum albumin
c	concentration
CCh	carbachol
CH_2Cl_2	dichlormethane
CHO-cells	chinese hamster ovary cells
CNS	central nervous system
COSY	correlated spectroscopy
CRC	concentration response curve
d	doublet
DEA	diethylamine
DIPEA	*N,N*-diisopropylethylamine
DMF	*N,N*-dimethylformamide
DMSO	dimthylsulfoxid
δ	chemical shift
EDC × HCl	N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride
ECL1	extracellular loop 2
eq.	equivalent(s)
ESI	electrospray ionization
EtOH	ethanol
FACS	fluorescence activated cell sorter
Fmoc	9-fluorenylmethoxycarbonyl
GPCR	G-protein coupled receptor

HBTU	O-(1H-benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HMBC	heteronuclear multiple bond correlation
HOBt	1-hydroxybenzotriazole hydrate
HRMS	high resolution mass spectrometry
HSQC	heteronuclear multiple bond correlation
J	coupling constant
k	retention (or capacity) factor (HPLC)
K_d	dissociation constant obtained from a saturation binding experiment
K_i	dissociation constant obtained from a competition binding experiment
K_2CO_3	potassium carbonate
m	multiplet
M	molar (mol/L)
MeOH	methanol
miniG	engineered minimal G-protein chimeras
MR	muscarinic receptor
MxR	muscarinic Mx (x = 1-5) receptor
NLuc	Nanoluceriferase
NlucC	C-terminal, smaller NLuc fragment with 11 amino acids
NlucN	N-terminal, larger NLuc fragment with 158 amino acids
NMS	N-methylscopolamine
PBS	phosphate buffer saline
Pd/C	palladium on carbon
PE	petroleum ether
pIC50	negative logarithm of the IC_{50} in M
pK_d	negative logarithm of the K_d in M
pK_i	negative logarithm of the K_i in M
ppm	parts per million

PyBOP	(Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
q	quartet
RP	reverse-phase
Rt	room temperature
s	singulet
SEM	standard eror of the mean
$SOCl_2$	thionyl chloride
t_0	hold-up time (also referred as dead time)
t	(1) time, (2) triplet
TFA	trifluoroacetic acid
TLC	thin-layer chromatography
t_R	retention time
UV	ultraviolet

Overview of bold compound numerals and lab codes

Compound	Labcode	Compound	Labcode	Compound	Labcode
2	UR-AP138	**54**	UR-CG152	**99**	UR-CG095
3	UR-AP148	**55**	UR-CG153	**100**	UR-CG269
4	UR-AP158	**56**	UR-CG154	**101**	UR-CG096
5	UNSW-MK259	**57**	UR-CG155	**102**	UR-CG190
8	UR-CG002	**58**	UR-CG172	**103**	UR-CG191
9	UR-CG057	**59**	UR-CG173	**104**	UR-CG197
10	UR-CG060	**60**	UR-CG195	**105**	UR-CG276
11	UR-CG025	**61**	UR-CG040	**106**	UR-CG277
12	UR-CG098	**62**	UR-MK242	**107**	UR-CG220
13	UR-CG079	**63**	UR-CG051	**108**	UR-CG221
17	UR-CG137	**64**	UR-CG263	**109**	UR-CG239
18	UR-CG138	**65**	UR-CG237	**110**	UR-CG213
19	UR-CG121	**68**	UR-CG156	**111**	UR-CG214
20	UR-CG140	**69**	UR-CG157	**112**	UR-CG215
21	UR-CG142	**70**	UR-CG160	**113**	UR-CG238
22	UR-CG122	**71**	UR-CG254	**114**	UR-CG235
23	UR-CG065	**72**	UR-SK-III-19	**115**	UR-CG104
24	UR-CG178	**73**	UR-CG103	**116**	UR-CG201
25	UR-CG128	**74**	UR-CG113	**117**	UR-CG202
27	UR-CG038	**75**	UR-CG114	**118**	UR-CG231
28	UR-CG039	**76**	UR-CG115	**119**	UR-CG203
30	UR-CG054	**77**	UR-CG150	**120**	UR-CG247
31	UR-CG055	**78**	UR-CG151	**121**	UR-CG233
33	UR-CG056	**79**	UR-CG170	**122**	UR-CG174
34	UR-CG061	**80**	UR-CG171	**123**	UR-CG175
35	UR-CG064	**81**	UR-CG194	**124**	UR-CG186
37	UR-CG179	**82**	UR-CG259	**125**	UR-CG187
38	UR-CG209	**83**	UR-CG041	**126**	UR-CG232
39	UR-CG216	**84**	UR-CG268	**127**	UR-CG240
40	UR-CG217	**85**	UR-CG097	**128**	UR-CG242
41	UR-SK-II-68	**86**	UR-CG100	**129**	UR-CG243
42	UR-CG206	**87**	UR-CG176	**130**	UR-CG244
43	UR-CG208	**88**	UR-CG177	**131**	UR-CG245
44	UR-CG083	**89**	UR-CG188	**132**	UR-CG248
45	UR-CG082	**90**	UR-CG189	**133**	UR-CG249
46	UR-CG081	**91**	UR-CG205	**134**	UR-CG250
47	UR-CG084	**92**	UR-CG260	**135**	UR-CG251
48	UR-CG080	**93**	UR-CG275	**137**	UR-CG265
49	UR-CG158	**94**	UR-CG117	**138**	UR-CG266
50	UR-CG159	**95**	UR-CG120	**139**	UR-CG267
51	UR-CG161	**96**	UR-CG092	**140**	UR-CG007
52	UR-MK216	**97**	UR-CG093	**141**	UR-CG008
53	UR-CG118	**98**	UR-CG094	**142**	UR-CG009

Compound	Labcode	Compound	Labcode	Compound	Labcode
143	UR-CG010	**148**	UR-CG020	**165**	UR-CG073
144	UR-CG015	**149**	UR-CG021	**166**	UR-CG074
145	UR-CG016	**156**	UR-SK-68	**167**	UR-AP175
146	UR-CG017	**157**	UR-MK257	**168**	UR-CG135
147	UR-CG018	**164**	UR-CG072	**169**	UR-MK342

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Einige der experimentellen Arbeiten wurden in Zusammenarbeit mit anderen Institutionen und Personen durchgeführt. Vermerke zu den Beiträgen der betreffenden Personen finden sich in den jeweiligen Kapiteln.

Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, 22.10.2020

_____

Corinna Weinhart