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PÄIVI KIVIRANTA

Design and Synthesis of Silent Information Regulator Human Type 2 (SIRT2) Inhibitors

Doctoral dissertation

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ABSTRACT

Silent information regulator human type 2 (SIRT2) enzyme belongs to the class III histone deacetylases (HDAC). It is one of the seven human yeast *Saccharomyces cerevisiae* Sir2 homologues. Sir2 enzyme and its homologues are also called sirtuins. Sirtuins need nicotinamide adenine dinucleotide (NAD^+) as a cofactor to be able to deacetylate histones and non-histone proteins. NAD^+ participates in the deacetylation reaction, in which the nicotinamide-ribosyl bond cleavage followed by transfer of the acetyl group of an acetylated lysine residue to ADP-ribose results; in free nicotinamide, deacetylated lysine and 2´- and 3´-*O*-acetyl-ADP-ribose. Nicotinamide is the endogenous inhibitor of sirtuins which regulates the sirtuin activity by the nicotinamide exchange reaction.

A highly potent and selective SIRT2 inhibitor would be useful for the study of the biological function of SIRT2. During the recent years, several research groups have been actively developing new SIRT2 inhibitors. The IC_{50} values of the most potent inhibitors range between high nanomolar and low micromolar levels. There are still a rather limited number of SIRT2 inhibitors. However, the structural diversity of the SIRT2 inhibitors and the molecular docking results suggest that SIRT2 inhibitors can interact with different binding sites of the enzyme. A couple of the most potent SIRT2 inhibitors have been tested on the preliminary disease models. SIRT2 might have a role in glioma tumorigenesis and Parkinson's disease. The inhibition of *N*-(5-quinolyl)propenamide (AGK2) has been reported to rescue α-synuclein toxicity and protect against dopaminergic cell death both biological changes caused by Parkinson's disease, thus tenovins-1 and -6 have been claimed to delay the tumour growth without a general toxicity.

The aim of the present study was to design and synthesize new SIRT2 inhibitors. A series of *N,N′*-bisbenzylidenebenzene-1,4-diamine and *N,N′*-bisbenzylidenenaphthalene-1,4-diamine derivatives were synthesized based on two earlier reported hits from molecular modelling and virtual screening. The most potent compound was *N,N′*-bis(2-hydroxybenzylidene)benzene-1,4-diamine, which was equipotent with the most potent hit compound and well-known SIRT2 inhibitor sirtinol.

A series of *N*-(3-(4-hydroxyphenyl)-propenoyl)-amino acid tryptamides was also based on the hit found by molecular modelling. The series was designed to study if the molecular size of the compound could be reduced. The most potent compounds, *N*-(3-(4-hydroxyphenyl)-propenoyl)-2-aminoisobutyric acid tryptamide and *N*-(3-(4-hydroxyphenyl)-propenoyl)-L-alanine tryptamide, were equipotent, 30% smaller in molecular weight, and slightly more selective (SIRT2/SIRT1) than the parent compound. The research of this new interesting backbone was continued and the structure-activity relationships were studied by different replacements in the original hit structure. As a result, the *N*-(3-phenylpropenoyl)-glycine tryptamide backbone was also a good backbone for SIRT2 inhibitors, and the series of compounds included several potent SIRT2 inhibitors. In addition, the series of compounds gave valuable tools for molecular modellers to study the binding interactions with SIRT2.

The known SIRT2 substrates were utilized to study SIRT2 substrate-based inhibitors. A series of thioacetylated tri-, tetra-, and pentapeptides based on the α-tubulin and p53 protein sequences were shortest peptide sequences published so far with SIRT1 and SIRT2 inhibitory activity. The most potent peptides had the inhibitory activities on high nanomolar (SIRT1) or low micromolar (SIRT2) level. In addition, two of the p53–based peptides were more selective SIRT1 inhibitors than well-known EX-527.

The present study provided new SIRT2 inhibitor backbones and introduced several potent SIRT2 inhibitors targeting different binding sites of the enzyme.

National Library of Medicine Classification: QU 62, QU 68, QU 136, QU 143, QV 744
Medical Subject Headings: Histone Deacetylases; Sirtuins; Enzyme Inhibitors; Enzyme Inhibitors / chemical synthesis; Amides / chemical synthesis; Peptides / chemical synthesis; Tubulin; Structure-Activity Relationship; Drug Design

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Kuopio, November 2008

Päivi Kiviranta

ABBREVIATIONS

AceCS2	Acetyl-coenzyme A synthetase 2
ACN	Acetonitrile
ADPR	ADP-ribose
O-AADPR	*O*-Acetyl-ADP-ribose
APT	Attached proton test
Boc	*tert*-Butoxycarbonyl
BOP	Benzotriazol-1-yloxytris-(dimethylamino)-phosphonium hexafluorophosphate
Cbz	Benzyloxycarbonyl
CR	Calorie restriction
Crm1	Chromosomal region maintenance 1
Cdk1	Cyclin-dependent kinase 1
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
ESI-MS	Electron spray ionization mass spectrometry
FID	Free-induction decay
FoxO	Forkhead box O transcription factor
HDAC	Histone deacetylase
HPLC	High performance liquid chromatography
HoxA	Homeobox A transcription factor
Hst2	Archeal Sir2 homologue of yeast
IC_{50}	Inhibitor concentration at which the enzyme reaction velocity is 50% of the uninhibited reaction
m/z	Mass-to-charge ratio
MS	Mass spectrometry
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced form of NAD^+

NMR	Nuclear magnetic resonance
ppm	Parts per million
rac	Racemate
rt	Room temperature
SAOS2	Human osteoplastic cells
Sir	Silent information regulator
Sir2-Af1	Sir2 homologue of Archae-bacterium 1
Sir2-Af2	Sir2 homologue of Archae-bacterium 2
Sir2Tm	Sir2 homologue of thermophilic bacterium
SIRT	Silent information regulator human type
TEA	Triethylamine
THF	Tetrahydrofuran
TMS	Tetramethylsilane
TRPM2	Transient receptor potential melastatin-related channel 2
UV	Ultraviolet
w/o	Without

LIST OF ORIGINAL PUBLICATIONS

The present doctoral dissertation is based on the following original publications:

I Kiviranta PH, Leppänen J, Kyrylenko S, Salo HS, Lahtela-Kakkonen M, Tervo AJ, Wittekindt C, Suuronen T, Kuusisto E, Järvinen T, Salminen A, Poso A, Wallén EAA. *N,N'*-Bisbenzylidenebenzene-1,4-diamines and *N,N'-Bis*benzylidenenaphthalene-1,4-diamines as Sirtuin Type 2 (SIRT2) Inhibitors. Journal of Medicinal Chemistry 49: 7907-7911, 2006.

II Kiviranta PH, Leppänen J, Rinne VM, Suuronen T, Kyrylenko O, Kyrylenko S, Kuusisto E, Tervo AJ, Järvinen T, Salminen A, Poso A, Wallén EAA. *N*-(3-(4-Hydroxyphenyl)-propenoyl)-amino acid tryptamides as SIRT2 inhibitors. Bioorganic and Medicinal Chemistry Letters 17: 2448-2451, 2007.

III Kiviranta PH,[#] Salo HS, [#] Leppänen J, Rinne VM, Kyrylenko S, Kuusisto E, Suuronen T, Salminen A, Poso A, Lahtela-Kakkonen M, Wallén EAA. Characterization of the binding properties of SIRT2 inhibitors with a *N*-(3-phenylpropenoyl)-glycine tryptamide backbone. Bioorganic and Medicinal Chemistry 16: 8054-8062, 2008. [#] Equal contribution.

IV Kiviranta PH, Suuronen T, Wallén EAA, Leppänen J, Tervonen J, Kyrylenko S, Salminen A, Poso A, Jarho EM. N^{ε}-Thioacety-Lysine-Containing Tri-, Tetra-, and Pentapeptides as SIRT1 and SIRT2 Inhibitors. Manuscript, submitted.

In publication **III**, the molecular modelling part is a contribution of Heikki Salo and it is not included in thesis.

CONTENTS

1 INTRODUCTION

Histone proteins are located in nucleosomes packed with the DNA in all eukaryotes. The amino termini of the histones are rich in lysines and they stick out of nucleosomes (Luger and Richmond 1998). They offer sites of several reversible covalent modifications and lead gene expression or silencing fulfil the requirements of the cell (Gottschling 2000, Nightingale *et al.* 2006). Histone deacetylases (HDACs) are enzymes, which remove the acetyl groups from acetylated lysine residues. HDACs are divided into four classes. The class I, II, IV are similar to each other, for example, in their catalytic cores and inhibitors. The class III HDACs, the sirtuins, have no sequence similarity to other HDACs and these proteins have their unique requirement for nicotinamide adenine dinucleotide (NAD^+) to be able to function (Imai *et al.* 2000, Michan and Sinclair 2007, Smith *et al.* 2000).

There are seven mammalian sirtuins (SIRTs) (Frye 2000). SIRT2 was reported the first time in 1999 (Frye 1999). SIRTs deacetylate histone and non-histone proteins (Buck *et al.* 2004, North *et al.* 2003, Wang *et al.* 2007). SIRT2 is also an α-tubulin deacetylase, unlike its evolutionary ancestor the Archeal Sir2 homologue of yeast Hst2 and other SIRTs (North *et al.* 2003). SIRT2 has been linked to the regulation of the mitotic progression of the cell cycle (Dryden *et al.* 2003, Inoue *et al.* 2007, North and Verdin 2007b). SIRT2 might have a role in glioma tumorigenesis and Parkinson's disease (Hiratsuka *et al.* 2003, Outeiro *et al.* 2007). However, understanding the full biological role and the different mechanisms of actions still needs further studies. Development of positive and negative regulators of SIRT2 is under intensive investigation (Porcu and Chiarugi 2005). There are more and more compounds published which show an *in vitro* inhibitory activity for SIRT2. However, the connection of the *in vitro* activity and the cellular effects needs further clarification.

When the SIRT2 inhibitor project started in Autumn 2002, the crystal structure of SIRT2 and the first two low micromolar inhibitors (sirtinol and A3) had recently been published (Finnin *et al.* 2001, Grozinger *et al.* 2001). Computational methods were employed to search for new inhibitors with different chemical backbones and to study the interactions between the inhibitors and the enzyme. News about the preferred

substrates of SIRT2 was also published during the first year of the project (North *et al.* 2003). Both the computational methods and the substrates of the enzyme were used as starting points for the development of new inhibitors.

2 REVIEW OF LITERATURE

2.1 Nomenclature and classification

The story of silent information regulators (Sirs) started in mid-eighties when four Sir genes were found from yeast *Saccharomyces cerevisiae* (Ivy *et al.* 1986, Shore *et al.* 1984). These genes code for the Sir proteins, which were studied to be part of a system of transcriptional inactivation, or silencing, an important and highly conserved mechanism of gene regulation. Sir2 enzyme and its homologues, also called the sirtuins, are classified as class III histone deacetylases (HDAC). They are unrelated to class I, II, IV HDACs and have their unique requirement for nicotinamide adenine dinucleotide (NAD^+) as a substrate (Imai *et al.* 2000, Smith *et al.* 2000). SIRTs are Sir2-like human homologues and they consists of seven members (SIRT1-SIRT7) (Frye 1999, 2000).

2.2 Distribution of SIRT2

The Sir2 gene is evolutionarily conserved, and a number of Sir2 enzyme homologues are reported from Archeal species, prokaryotes and eukaryotes (Brachmann *et al.* 1995). The Sir2 homologues of mammals share a conserved central deacetylase domain but have different N- and C- termini (North and Verdin 2004). They also display distinct subcellular localization.

SIRT1 is nuclear (Frye 1999). SIRT2 was first reported to be a cytoplasmic enzyme, which was highly expressed in the heart, brain and skeletal muscles (Afshar and Murnane 1999, Perrod *et al.* 2001). Later, SIRT2 was observed to shuttle between the nucleus and cytoplasm and to localize to mitotic structures (Bae *et al.* 2004, Dryden *et al.* 2003, Hiratsuka *et al.* 2003, North *et al.* 2003, North and Verdin 2007a). SIRT3-5 are localized in mitochondria (Onyango *et al.* 2002, Schwer *et al.* 2002), SIRT6 is associated with heterochromatic regions, and SIRT7 is concentrated in the nucleolus (Michishita *et al.* 2005).

Further studies are needed for understanding the mechanism regulating the expression of the SIRT2 enzyme. It has been claimed that the SIRT2 enzyme activity could be regulated by phosphorylation in the nucleus and ubiquitination in the cytoplasm (Suzuki and Koike 2007a). Wilson *et al*. (2006) reported that the Archeal Sir2 homologue of

yeast Hst2 was exported into the cytoplasm by the chromosomal region maintenance 1 (Crm1) protein, and assumed, that the same active nuclear export exists also for SIRT2. This was confirmed a year later by two research groups (Inoue *et al.* 2007, North and Verdin 2007a). In the brain, the proteolipid protein DM20 provides the same active transport of SIRT2 into myelin of the central nervous system (Werner *et al.* 2007).

2.3 Structure

SIRT2 contains a catalytic core of 304 amino acids and a so-called N-terminal helical extension of 19 amino acids. The crystal structure of SIRT2 has been published in 2001 (Finnin *et al.* 2001). The crystal structure revealed that SIRT2 consists of two domains that are connected by four conserved loops of the polypeptide chain. The bigger domain is an NAD^+-binding domain, which is a variant of the Rossmann fold, a structural motif found in proteins that bind nucleotides (Bellamacina 1996). The other, smaller domain is composed of a helical module and a single zinc-binding module (figure 2.1). At the interface of the two domains is a large groove, which includes the NAD^+-binding site, and contains residues invariant across the Sir2 homologues. A pocket lined with hydrophobic residues has been found in the large groove, and it has been suggested to be the substrate binding site (Finnin *et al.* 2001). This hydrophobic region has been reported to be unique in the structures of SIRT2 and yeast Hst2 but absent from the structures of the archae-bacterium Sir2-Af1 complexed with NAD^+ and Sir2-Af2 bound to an acetylated p53 peptide (Avalos *et al.* 2002, Finnin *et al.* 2001, Min *et al.* 2001, Zhao *et al.* 2003).

The reported sequence comparison study with SIRT2 and Sir2-Af1 complexed with NAD^+ suggested that the large groove contains three sites for NAD^+-binding, called A, B, and C sites (figure 2.2) (Finnin *et al.* 2001). In the A site, amino acids of the enzyme have been reported to form hydrogen bonds with the 2′- and 3′-hydroxyl groups of the adenine ribose and to interact with the adenine base and phosphate oxygens. At the B site, two amino acids have been reported to bind by forming hydrogen bonds with the 3′-hydroxyl of the nicotinamide ribose. However, the large groove of SIRT2 in the B site is wider than that of Sir2-Af1 which makes the comparison difficult.

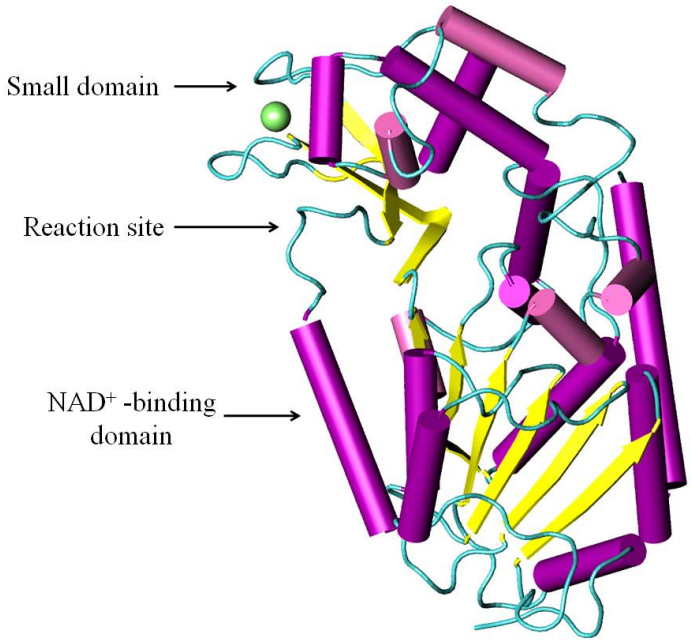


Figure 2.1. The structure of SIRT2. The active site of the SIRT2 enzyme is pointed by the arrow. Zinc is colored lime, α-helices are colored purple, and β-sheets are colored yellow. The picture was kindly created by M.Sc. Heikki Salo.

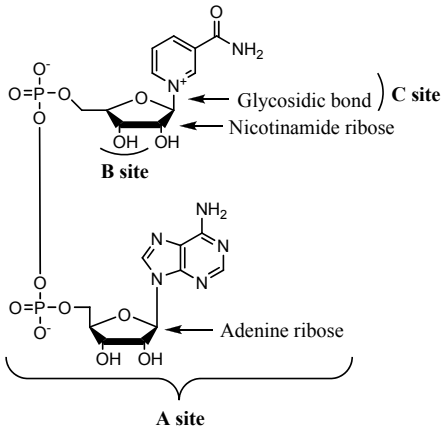


Figure 2.2. NAD^+ structure and its binding sites to the enzyme.

The flexible C site of SIRT2 has been claimed to be involved in the polarization and hydrolysis of the NAD^+ glycosidic bond and to show the HDAC activity. The B and C sites together are reported to form the active site of the enzyme (Finnin *et al.* 2001). These NAD^+-binding sites clarify the progress of the deacetylation reaction described later in this literature review.

2.4 Substrates

Several endogenous SIRT2 substrates have been reported in the literature (table 2.1). The study of Borra *et al.* (2004) have reported that SIRT2 has the strongest substrate preference for lysine-8, -12, and -16 of histone H4 tested on *in vitro* HPLC based assay (Borra and Denu 2003, Borra *et al.* 2002). Several monoacetylated histone H3 and H4 peptides were employed in the assay based on the knowledge that they are *in vivo* acetylation sites (Csordas 1990, Loidl 1994). The substrate preference of SIRT2 for lysine-16 of H4 was also shown in the study of Vaquero *et al.* (2006). SIRT2 was reported to deacetylate lysine-16 of H4 and lysine-9 of H3 *in vitro* on HDAC based assays followed by Western blots, although only lysine-16 of H4 was shown to be a valid substrate in 293 cells (Vaquero *et al.* 2006). However, α-tubulin has been claimed to be a preferred substrate of SIRT2 due to SIRT2s predominantly cytoplasmic location and co-localization with microtubules (North *et al.* 2003). Another member of the histone deacetylase family, HDAC6, has also been indentified to function as a α-tubulin deacetylase (Hubbert *et al.* 2002).

Recently, a study with HEK293 cells has been reported where the interaction of SIRT2 with the cellular regulatory proteins 14-3-3 β and γ has shown to regulate the transcriptional activity of p53 protein by deacetylation (Jin *et al.* 2008). In addition, SIRT2 deacetylation mechanism has been connected to the Forkhead box O (FoxO) transcription factors tested on the several cell cultures (Daitoku *et al.* 2004, Jing *et al.* 2007, Wang *et al.* 2007). It has been speculated, that since Sir2-like proteins have been conserved in evolution, they might have been required to perform diverse deacetylation reactions on a wide variety of substrates in a manner regulated by cellular energy and redox states (Smith *et al.* 2000). However, it has been suggested that the sirtuins

recognize their substrates based on certain amino acid side chains near the N^{ε}-acetyl lysine side chain (Cosgrove *et al.* 2006, Garske and Denu 2006).

Table 2.1. Endogenous substrates of SIRT2 and their reported functions.

Substrate	Type of cells tested	Function	Reference
Lys16 of H4	in 293 cells	chromatin condensation	(Vaquero *et al.* 2006)
Lys40 of α-tubulin	in HeLa cells	cell cycle	(North *et al.* 2003)
FoxO1	in HepG2, HEK293 and HEK293T cells	transcription	(Daitoku *et al.* 2004)
	in 3T3-L1 adipocytes	adipocyte differentiation	(Jing *et al.* 2007)
FoxO3a	in HEK293T cells	oxidative stress caloric restriction	(Wang *et al.* 2007)
p53	in HEK293 cells	transcription	(Jin *et al.* 2008)

2.5 Deacetylation reaction

In the beginning of the research of SIRT2, it was unclear if SIRT2, among with the other human sirtuins, is an ADP-ribosyl transferase (Frye 1999) or a histone deacetylase (Smith *et al.* 2000). Imai *et al.* (2000) reported already back then, that the human sirtuins catalyze both reactions. Later on, it has been concluded that the histone ADP-ribosyl transferase activity is a side reaction of the deacetylation reaction (Tanner *et al.* 2000) in which the histone deacetylase activity is at least 1000-fold higher than the ADP ribosylation activity (Finnin *et al.* 2001).

2.5.1 Reaction stoichiometry

In Archeal species, prokaryotes and eukaryotes Sir2 enzyme homologues have been reported to have a NAD^+-dependent deacetylase activity (Imai *et al.* 2000, Landry *et al.* 2000b, Smith *et al.* 2000). The identification of the unique reaction product *O*-acetyl-ADP-ribose (*O*-AADPR) resolved the reaction stoichiometry. In the reaction, one

molecule of NAD^+ is hydrolyzed to nicotinamide and *O*-AADPR for every molecule of acetylated lysine that is deacetylated (Landry *et al.* 2000a, Tanner *et al.* 2000, Tanny and Moazed 2001).

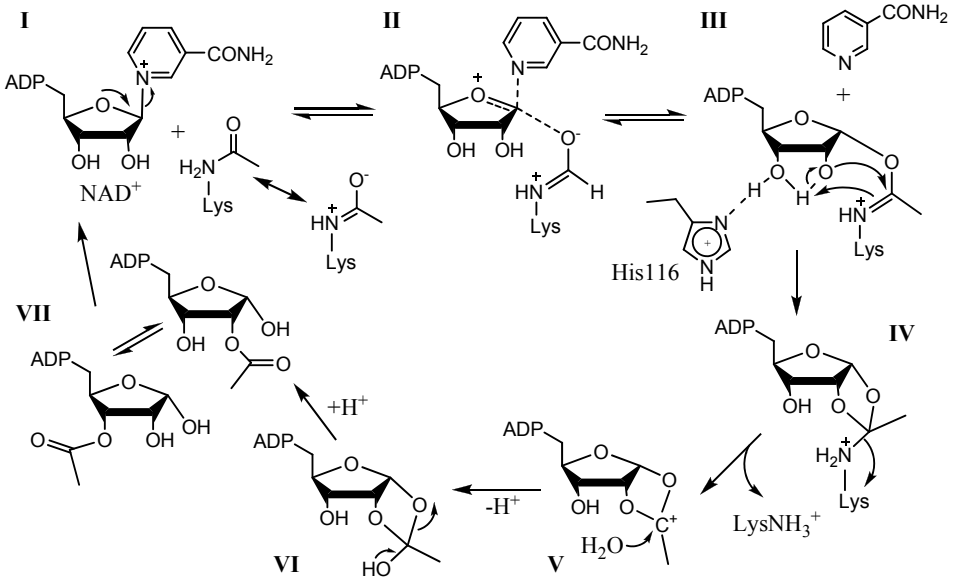
2.5.2 Reaction mechanism

The Sir2 deacetylation reaction mechanism has been under wide discussion in the literature. Three reaction mechanisms have been; 1) the nucleophilic mechanism (Tanny and Moazed 2001), 2) the enzyme nucleophile mechanism (Landry *et al.* 2000a, Min *et al.* 2001, Tanner *et al.* 2000) and 3) the ADP-ribose-peptidyl-imidate mechanism (Sauve *et al.* 2001). The ADP-ribose-peptidyl-imidate mechanism explains the best the chemistry of sirtuins and the available experimental data (scheme 2.1) (Sauve *et al.* 2006).

The reaction of sirtuins has been reported to start when the carbonyl oxygen of the acetyl group of lysine is in the right position relative to NAD^+. The electrophilic capture of an acetyl oxygen by ADP-ribose (ADPR) is formed and stabilized by the enzyme. The proposed capture requires a highly electrophilic ADPR, which gives the weakness of the amide as a nucleophile (Sauve *et al.* 2001). The mechanism of nicotinamide-ribosyl bond cleavage has been reported to proceed via a S_N2-like mechanism (I→II→III) (Smith and Denu 2007c). The transition state couple is named as an oxacarbenium-ion transition state (complex II) and an ADPR-peptidyl imidate (complex III). The reaction has been reported to be written as a reversible step. The step clarifies nicotinamide reactivity through a base exchange to reform NAD^+. The imidate (complex III) has a long enough lifetime to equilibrate nicotinamide in the reaction site nicotinamide pocket and reform NAD^+ by the reversal reaction (Sauve and Schramm 2003). This is an important observation since age-related human diseases seem to have a connection to the changes in the NAD^+ level (Lin and Guarente 2003).

According to earlier literature, this step of the reaction was suggested to be a reaction checkpoint where concentration of nicotinamide in a cell reverses or forwards the NAD^+-dependent reaction (Sauve *et al.* 2001). However, it has later been reported that the nucleophilic attack of the acetylated lysine oxygen at the 1′-position of the nicotinamide ribose of NAD^+ forms the covalent intermediate α-1′-*O*-alkylamidate.

According to this mechanism, the final product, 2′- and 3′-*O*-AADPRs, release would be the rate limiting step of the reaction (Borra *et al.* 2004, Smith and Denu 2006).



Scheme 2.1. The ADPR-peptidyl-imidate mechanism (modified from Sauve *et al.* 2006).

When the ADPR-peptidyl-imidate mechanism proceeds further, the deacetylation reaction is continued by activation of the 2′-hydroxyl group of ribose through the 3′-hydroxyl group of ribose and the His116 at the reaction site (complex III). The function of His116 as a proton acceptor has been confirmed by the His to Ala mutation in which the enzymatic activity of Sir2-Af1 was lost (Min *et al.* 2001). In addition, the activation of the 2′-hydroxyl group of ribose has been confirmed by NMR studies (Jackson and Denu 2002). The nucleophilic attack of the 2′-hydroxyl group of ribose on the imidate forms the intermediate IV. Lysine is eliminated to form an oxonium intermediate (complex V). The oxonium intermediate has been reported to capture water to form a tetrahedral intermediate VI. The 1′-hydroxyl group is eliminated resulting in an equilibrium of the 2′- and 3′-*O*-acetyl-ADP-ribose isomers as the final product on the enzyme (complex VII). The final reaction steps (V→VII) have been studied by

reactions conducted in ^{18}O labelled water (Sauve *et al.* 2001) and by NMR studies (Jackson and Denu 2002).

2.6 Regulation of SIRT activity

It has been reported that NAD^+ can bind to the sirtuins in different conformations. Studies with both Sir2-Af1 and Sir2-Af2 complexed with NAD^+ have suggested that the binding site of a nicotinamide of NAD^+ is at the conserved C site and the acetyl-lysine substrate is bound to its binding tunnel, that intersects the large groove (Avalos *et al.* 2004, Min *et al.* 2001). In this so-called productive conformation of NAD^+, the nicotinamide ring and ADPR are in correct relative positions with the catalytically important residues. The productive binding of NAD^+ has been reported to be induced by the binding of the acetyl-lysine substrate (Avalos *et al.* 2004). This has been confirmed with the isothermal titration calorimetry study, which was not able to detect the binding of NAD^+ in SIRT2. Due to the result, the acetyl-lysine substrate seems to bind before NAD^+. It has also been suggested that NAD^+ and the acetyl-lysine substrate must form a ternary complex prior to catalysis (Borra *et al.* 2004).

However, studies of the so-called non-productive conformations with NAD^+ have suggested that these conformations are energetically lower when the acetyl-lysine substrate is not bound. The binding of the acetyl-lysine substrate to Sir2 causes the favourable conformational change for productive NAD^+ binding (Avalos *et al.* 2004). It is therefore likely that the large groove of SIRT2 undergoes a conformational change upon binding of the acetyl-lysine substrate or NAD^+ (Avalos *et al.* 2002).

The interactions of nicotinamide with the C site in the productive conformation of NAD^+ have been reported to induce the destabilization of NAD^+ which favours the nucleophilic attack of the carbonyl oxygen of the acetyl group on the ribose (I → II, scheme 2.1). The reported products are nicotinamide and the α-1´-*O*-alkylamidate intermediate which has been claimed to be located between the acetyl-lysine binding tunnel and the A and B sites of the NAD^+ binding cleft (figure 2.2). At this point, the deacetylation reaction can proceed to the final products or, depending on the nicotinamide concentration, to the nicotinamide exchange reaction (Avalos *et al.* 2004). The nicotinamide exchange reaction, which regulates the sirtuin activity by

nicotinamide inhibition (Bitterman *et al.* 2002), is a competing reaction with the deacetylation reaction (Sauve and Schramm 2003) described later in this literature review.

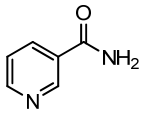
The final products of the deacetylation reaction are nicotinamide, the deacetylated lysine-enzyme and 2′- and 3′-*O*-AADPRs (Jackson and Denu 2002, Sauve *et al.* 2001). When the deacetylation reaction was reported, it was suggested that the unique product *O*-AADPR has an important signalling role (Tanner *et al.* 2000). It has later been reported that *O*-AADPR has a delay effect on embryo cell division in blastomeres (Borra *et al.* 2002) and an activating effect on the cytoplasmic domain of the transient receptor potential melastatin-related channel 2 (TRPM2), which is a nonselective cation channel, whose prolonged activation leads to cell death (Grubisha *et al.* 2006). In addition, the regulation by nicotinamide may function on exact opposite ways in different species since it has been reported that nicotinamide extends the replicative lifespan of primary human fibroblasts (Lim *et al.* 2006) whereas it shortens the replicative lifespan of *Saccharomyces cerevisiae* (Bitterman *et al.* 2002).

2.6.1 Inhibition of SIRT

The nicotinamide exchange reaction is a competing reaction with the deacetylation reaction (Sauve and Schramm 2003). It regulates the sirtuin activity by nicotinamide inhibition (Bitterman *et al.* 2002), which was also confirmed for SIRT2 (Jackson *et al.* 2003). Concentrations of nicotinamide **1** in mammalian tissues have been reported to vary between 11–400 μM (Bitterman *et al.* 2002). The reported observation with mouse Sir2 has claimed that the mammalian enzymes might be subjected to stronger regulation by nicotinamide **1** than yeast and bacterial Sir2s (Sauve and Schramm 2003). Reported IC_{50} value of nicotinamide **1** is 100.5 μM for SIRT2 tested in the [^{3}H]-substrate based assay (Tervo *et al.* 2004). Nicotinamide has been reported to function as a noncompetative inhibitor against NAD^+ (Bitterman *et al.* 2002, Landry *et al.* 2000a) and the acetylated substrate for Sir2-like enzymes (Borra *et al.* 2004).

It has been claimed that the great flexibility of the Sir2 structure facilitates the nicotinamide exchange in and out the enzyme. Nicotinamide might reform β-NAD^+ by doing a reverse attack on the β-face of the α-1′-*O*-alkylamidate intermediate (Avalos *et*

al. 2004). The site of the nicotinamide inhibition was confirmed by the single point mutation study with Sir2Af2 and Sir2 homologue of thermophilic bacterium Sir2Tm which directed a dual role for the C site of the groove in both the nicotinamide exchange and the deacetylation reaction (Avalos *et al.* 2005).



1 Nicotinamide

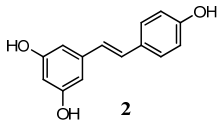
It has been reported that compounds that can interact with the C site and prevent NAD^+ from adopting its productive conformation could act as competitive inhibitors. Thus, compounds which are able to participate in the possible flipping mechanism and react with the α-1′-*O*-alkylamidate intermediate, could act as noncompetative inhibitors (Avalos *et al.* 2005). It has also been suggested that compounds that could mimic the binding of the acetyl-lysine substrate might be potent and selective inhibitors of Sir2 deacetylases over other NAD^+-metabolizing enzymes (Smith and Denu 2007c). In addition, it has been suggested that small molecule regulation of sirtuins involves the cellular balance of NAD^+ to nicotinamide (Grubisha *et al.* 2005), and this would be controlled by enzymes involved in NAD^+ synthesis or salvage (Denu 2003, Lin and Guarente 2003).

2.6.2 Activation of SIRT

The positive regulator could enhance the deacetylation reaction of SIRTs. The function of Sir2 activators has been postulated to occur by blocking the binding site of free nicotinamide (Marmorstein 2004). It has been reported that the yeast life span can be extended through the action of calorie restriction by increasing the activity of Sir2 (Lin *et al.* 2000). The most potent small molecule activator called resveratrol **2** has been reported to mimic the calorie restriction and assist the life span extension in yeast and increase cell survival by stimulating SIRT1-dependent deacetylation of p53 (Howitz *et al.* 2003). However, it has later been reported that the activation was caused by a specific substrate containing a non-physiological, fluorescent Fluor de Lys moiety

(Kaeberlein *et al.* 2005a). More accurately, it was the fluorophore of that substrate, which posed the substrate bind more tightly to SIRT1 in the presence of resveratrol **2** (Borra *et al.* 2005).

SIRT2 activation by resveratrol **2** has not been observed in a fluorescence based Fluor de Lys kit (Borra *et al.* 2005). However, the study of slow Wallerian degeneration mice has later suggested that resveratrol **2** abolishes the resistance to axonal degeneration by enhancing SIRT2-mediated tubulin deacetylation. An activation mechanism of resveratrol **2** was reported to be unknown (Suzuki and Koike 2007b).



2

2.7 Biological relevance of SIRT2

The biological function of SIRT2 appears to be largely unknown. Although, the yeast Sir2 gene is related to the human SIRT2 gene and its functions are widely studied, the functions of these two homologues do not seem to be related (Afshar and Murnane 1999, Guarente 2000). However, the aim of this chapter is to give an overview of the reported biological functions of the SIRT2 enzyme and the relevant prospects on cell cycle regulation and some human diseases.

2.7.1 Tubulin deacetylation

North *et al.* (2003) have published a highly cited article where they showed that the SIRT2 enzyme deacetylates lysine-40 of α-tubulin both *in vitro* and *in vivo*. This has not been reported for other human sirtuins or yeast Hst2. SIRT2 has been claimed to function together as a complex with HDAC6, another HDAC with α-tubulin deacetylase activity, in cytoplasm (Hubbert *et al.* 2002, North *et al.* 2003). The microtubule network is formed from α- and β-tubulin heterodimers and play a crucial role in the regulation of cell shape, intracellular transport, cell mobility and cell division (Nogales 2000). Recently, the suggested complex was confirmed by the report that tubulin binds only to the HDAC6-SIRT2 complex, not either of HDACs individually. The study was done

with the human osteoplastic cells (SAOS2) (Nahhas *et al.* 2007). However, SIRT2 and HDAC6 are expressed in several cell types and it has been suggested that in the nervous system they are localized to different cell types and are unlikely form the complex *in vivo* (Southwood *et al.* 2006).

2.7.2 Histone deacetylation

SIRT2 has been reported to deacetylate lysines-8, -12, and -16 of histone H4 *in vitro* (Borra *et al.* 2004). Earlier, it was postulated that SIRT2 may influence silencing without being targeted to the site of repression (Perrod *et al.* 2001). Later, it has been reported that there is a correlation between SIRT2 and the level of acetylated lysine-16 of histone H4 in the nucleus during the G_2 to M transition and mitosis of the cell cycle. This means that mainly cytoplasmic SIRT2 can move to the nucleus and is associated there with chromatin (Vaquero *et al.* 2006).

2.7.3 Cell cycle regulation

The cell cycle involves four phases: the cell grows (G1), the DNA is replicated (S), the cell prepares to divide (G2) and the cell divides (M) (figure 2.3) (Alberts *et al.* 2002). Many studies have reported that SIRT2 participates in these phases of the cell cycle in several types of cells. However, the targets for SIRT2 are so far largely unknown. The understanding of the involvement of SIRT2 in the cell cycle regulation processes can lead to the new therapeutic possibilities.

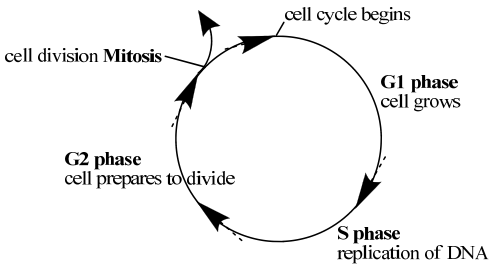


Figure 2.3. The cell cycle (modified from Alberts *et al.* 2002).

It seems to be clear that SIRT2 regulates mitotic progression (M) (Dryden *et al.* 2003, Inoue *et al.* 2007, North and Verdin 2007b). SIRT2 is phosphorylated both *in vitro* and *in vivo* on Ser368 by a mitotic cell cycle regulator, a cyclin-dependent kinase 1 (Cdk1). SIRT2 phosphorylation mediates a delay in mitosis (North and Verdin 2007b). Furthermore, the study in glioma cell lines provided evidence for that SIRT2 may function as a novel mitotic checkpoint enzyme in the early metaphase (M) to prevent chromosomal instability (Inoue *et al.* 2007). SIRT2 phosphorylation and expression has also been reported to increase during the G_2 and M phases and to have a role in the control of the G2 to M transition in SAOS2 cells (Dryden *et al.* 2003). SIRT2 may directly influence chromatin condensation during the G_2 to M transition by regulating deacetylation of lysine-16 of histone H4 (Vaquero *et al.* 2006). It was recently reported that SIRT2 decreases the transcriptional activity of p53 through the interaction with 14-3-3 β and γ proteins (Jin *et al.* 2008). In addition, SIRT2 has also been reported to interact with several transcription factors Homeobox A10 (Hoxa10), FoxO1 and FoxO3a in different mammalian cells (Bae *et al.* 2004, Jing *et al.* 2007, Wang *et al.* 2007). SIRT2 has even been connected to cell death in response to various stress stimuli including DNA damage (Matsushita *et al.* 2005).

2.7.4 Aging and aging related diseases

The important factors in the control of the aging process are calorie restriction, insulin-like signalling pathway and oxidative stress resistance. The connection between these factors and SIRT2 has been under intense research (Michishita *et al.* 2005, Wang *et al.* 2007). It has been reported that the life span of yeast can be extended through the action of calorie restriction (CR) which has been linked to Sir2 by decreasing NADH levels. NADH (the reduced form of NAD^+) is a competitive inhibitor of Sir2 (Guarente and Picard 2005, Lin *et al.* 2004). It has also been reported that the levels of SIRT1 are increased under CR conditions in rats (Cohen *et al.* 2004), although further studies have postulated that CR-induced lifespan might occur independently of the mammalian SIRTs (Kaeberlein *et al.* 2005b, Longo 2008, Tsuchiya *et al.* 2006). These controversial issues on the connection between the effect of CR on longevity in mammals and SIRTs should be studied further.

SIRT2 has been reported to be the most abundant sirtuin in adipocytes. SIRT2 deacetylation has been claimed to regulate adipocyte differentiation through FoxO1 transcription factor (Jing *et al.* 2007) and oxidative stress and CR through FoxO3a transcription factor in the cytoplasm (Wang *et al.* 2007). Hence, regulators of SIRT2 could provide potent drugs for obesity and its complications (Jing *et al.* 2007).

There are a few early studies, which have reported the role of SIRT2 in neurodegenerative diseases. Li *et al.* (2007) and Tang and Chua (2008) have presented the possible connection between SIRT2 and brain aging and related diseases. SIRT2 has been reported to regulate oligodendroglial differentiation and maturation through its tubulin deacetylation activity. The main function of oligodendroglia is the insulation of the axons in the central nervous system. Selective, small-molecule inhibitor of SIRT2 has been reported to protect against α-synuclein-mediated toxicity in cellular models of Parkinson's disease (Outeiro *et al.* 2007). The suggested inhibition mechanism claimed that SIRT inhibition could reduce the formation of abnormal protein aggregates inside nerve cells. These aggregates are called Lewy bodies and they contain α-synuclein (Outeiro *et al.* 2007). This study together with other recent publications suggest that SIRT2 might function as a negative regulator of biological stress (Grubisha *et al.* 2006, Lynn *et al.* 2008, Outeiro *et al.* 2007). In addition, a study with wild-type granule cells suggested that SIRT2 inhibition might enhance microtubule acetylation and resistance to axonal degeneration (Suzuki and Koike 2007a).

The anticancer activity of class I and II HDAC inhibitors is well known. One of the latest accepted compounds called SAHA (Zolinda®) is used for treatment of cutaneous T-cell lymphoma (Mann *et al.* 2007). Thus, the interest of the anticancer activity of SIRT2 inhibitors has been under intense research. However, one should bear in mind, that compounds able to inhibit class I and II HDACs are ineffective in inhibiting the sirtuins (Mai 2007) due to the different type of catalytic mechanism (Imai *et al.* 2000, Smith *et al.* 2000). It has been reported that the human SIRT2 gene is located at the region of the genome, 19q13.1 (Voelter-Mahlknecht *et al.* 2005), that is frequently deleted in human gliomas and gastric carcinomas, supporting its potential role as a gene that protects cells from excessive proliferation and, further, cancer (Hiratsuka *et al.* 2003, Inoue *et al.* 2007). SIRT2 down-regulation in tumors has also been reported with

human brain tumor cell lines and chicken DNA-damaged cells (Matsushita *et al.* 2005, Voelter-Mahlknecht *et al.* 2005). This may offer a novel tumor marker (Wang *et al.* 2007). In addition, SIRT2 has been reported to interfere with cell adhesion in the cervical cancer cell line (Pandithage *et al.* 2008).

SIRT2 has also been reported to have a role in mediating cell survival in cardiac myocytes (Alcendor *et al.* 2004) and to have a reduced expression in diabetic hearts (Turdi *et al.* 2007). It has also been suggested to interact with HoxA10 that may have a role in the development of adult reproductive tissues (Bae *et al.* 2004).

2.8 SIRT2 inhibitors

The aim of this section is to give an overview of published SIRT2 inhibitors and their structure-activity relationships. The inhibitory activities against other human SIRTs are also discussed if reported. Direct comparisons will be made only between inhibitors that have been tested in the same test system.

2.8.1 Small molecule inhibitors

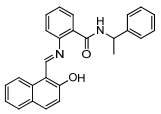
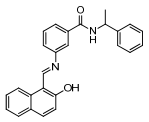
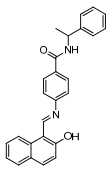
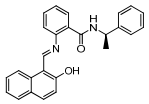
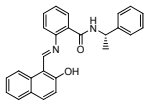
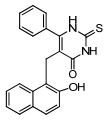
The first reported cell permeable SIRT2 inhibitor was sirtinol **3** found by a high throughput, phenotypic screen in cells (table 2.2). The inhibitory activity has been reported to be 38 μM tested *in vitro* in a radioactivity based HDAC assay (Grozinger *et al.* 2001). However, sirtinol has been reported to be poorly soluble and to precipitate in buffer and protein-containing solutions and during a crude enzyme preparation (Heltweg *et al.* 2003, Neugebauer *et al.* 2008).

The imine derived from 2-hydroxy-1-naphthaldehyde in the structure of sirtinol **3** was expected to be important for the inhibitory activity. However, it has been reported that 2-hydroxy-1-naphthaldehyde inhibits the SIRT2 activity alone only moderately (Grozinger *et al.* 2001). Removal of the 2-hydroxy group of sirtinol decreases the inhibitory activity of SIRT1 (Mai *et al.* 2005). The tested 2-hydroxy-1-naphthaldehyde derivatives have been reported to give IC_{50} values in the range 50–70 μM for SIRT2. However, the structures of the derivatives were not published (Grozinger *et al.* 2001).

Sirtinol **3** has also been tested in a fluorescence based HDAC assay, together with its derivatives **4**–7 (table 2.2). The IC_{50} value of sirtinol **3** was 57.7 μM and 131 μM for

SIRT2 and SIRT1, respectively. The *meta*- and *para*-isomers of sirtinol (**4** and **5**) were twice to ten-times more potent for both SIRT2 and SIRT1. (*R*)- and (*S*)-sirtinols (**6** and **7**) were equipotent and they could not bring out an enantioselective inhibitory effect (Mai *et al.* 2005). Sirtinol **3** has also been reported to have an *in vivo* effect on the acetylation status of p53 through SIRT1 in human endothelial cells (Ota *et al.* 2007).

Table 2.2. β-Naphthol analogues and their inhibitory activities for SIRT2 and SIRT1.

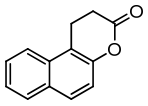
Compd	Name	Structure	IC_{50} (μM) SIRT2	IC_{50} (μM) SIRT1	Reference
rac-**3**	sirtinol		38 ± 2 [a]	60 [a]	(Grozinger *et al.* 2001)
			57.7 ± 9 [b]	131 ± 11 [b]	(Mai *et al.* 2005)
rac-**4**	*meta*-sirtinol		35.7 ± 2	59 ± 2	(Mai *et al.* 2005)
rac-**5**	*para*-sirtinol		25.9 ± 6	13 ± 2	(Mai *et al.* 2005)
(*R*)-**6**	(*R*)-sirtinol		49.3 ± 6	55 ± 5	(Mai *et al.* 2005)
(*S*)-**7**	(*S*)-sirtinol		39.4 ± 5	67 ± 4	(Mai *et al.* 2005)
8	cambinol		59 ± 4	56 ± 2	(Heltweg *et al.* 2006)

[a] Tested in a radioactivity based HDAC assay. [b] Tested in a fluorescence based HDAC assay.

The structurally similar β-naphthol analogue, cambinol **8** has been reported to be an equipotent SIRT2 and SIRT1 inhibitor with IC_{50} values of 59 μM and 56 μM,

respectively, tested in a radioactive based HDAC assay (table 2.2). Cambinol **8** is also inhibiting SIRT2 *in vivo*. The reported competition studies of SIRT2 with NAD^+ and histone H4-peptide substrates revealed that cambinol **8** is noncompetitive with NAD^+ but competitive with the substrate. Cambinol **8** is the first SIRT inhibitor, which has been reported to show an antitumor activity *in vitro* tested in a mouse xenograft model. This has suggested that SIRT inhibitors could be used as novel anticancer agents (Heltweg *et al.* 2006).

Splitomicin **9**, a β-naphthol analogue and a by-product in the synthesis of cambinol (Heltweg *et al.* 2006), has been reported to inhibit yeast Sir2 with an IC_{50} value of 60 μM in a yeast cell-based screen (Bedalov *et al.* 2001). Hydrolysis of the lactone ring of splitomicin and its analogues at neutral pH have complicated their studies in mammalian cells (Posakony *et al.* 2004a). However, several splitomicin analogues, such as compounds **10**–**13** in table 2.3, have been reported to be potent SIRT2 inhibitors (Neugebauer *et al.* 2008).



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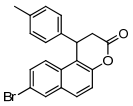
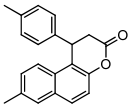
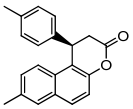
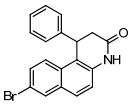
The series of compounds was tested in a homogenous deacetylase assay using a fluorescent lysine derivative, that was developed in the group of Heltweg *et al.* (2005). The results claimed that the bromo or methyl substituent in the 8-position of the naphthalene ring (**10**–**13**) had a positive effect on the inhibitory activity. In addition, replacing the lactone ring by a lactam ring resulted in compound **13** which had a similar inhibitory activity and an increased stability (Neugebauer *et al.* 2008).

The importance of the naphthalene ring has not been studied although it has not been required for the inhibitory activity in yeasts (Posakony *et al.* 2004b). Selected compounds were also tested for inhibition of proliferation of MCF-7 breast cancer cells, which confirmed the antitumor activity of SIRT2 inhibitors. Generally, these compounds were not potent cytotoxic agents on those cells which might due to the high lipophilicity of splitomicin derivatives. But compounds (**11**–**13**), which inhibited SIRT2

in the low micromolar region, were also the most potent antiproliferative agents (Neugebauer *et al.* 2008).

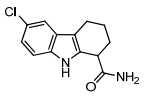
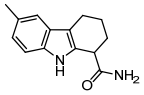
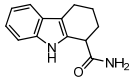
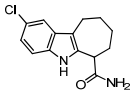
Table 2.3. Splitomicin derivatives and their inhibitory activities for SIRT2 (Neugebauer *et al.* 2008).

Compd	Structure	IC_{50} (μM) SIRT2
rac-**10**		1.5 ± 0.5
rac-**11**		1.5 ± 0.6
(*R*)-**12**		1.0 ± 0.3
rac-**13**		6.4 ± 0.3

A series of indoles are one of the most potent SIRT inhibitors reported so far (table 2.4). Compounds **14**–**16** were found by high-throughput screening for recombinant human SIRT1 and tested in a fluorescence based assay. Compound **17**, the seven-membered-ring analogue, has been synthesized as a ring modification of **14** (table 2.4). All compounds showed better inhibitory activity for SIRT1 than SIRT2. Compound **14** was 200-times more potent for SIRT1 than SIRT2. The inhibitory activity of **14** was 19.6 μM and 0.098 μM for SIRT2 and SIRT1, respectively. Thus, **14** is one of the most selective inhibitors (SIRT1/ SIRT2) reported. Small nonpolar groups at the 6-position in **14**, **15**, and **16** have not been claimed to have a significant effect on the inhibitory activity of SIRT2. Compound **16** was an equipotent SIRT1 inhibitor as compared to **14**, whereas **17** was seven times more potent for SIRT2 as compared to **14**. The enantiomers of **14** and **17** were separated but their inhibitory activities of SIRT2 have not been

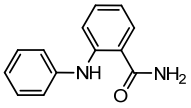
reported. Overall, the series of indoles have been reported to be low molecular weight, cell-permeable, orally bioavailable, and metabolically stable (Napper *et al.* 2005).

Table 2.4. Indoles **14**–**17** and their inhibitory activities for SIRT2 and SIRT1 (Napper *et al.* 2005).

Compd	Structure	IC_{50} (μM) SIRT2[a]	IC_{50} (μM) SIRT1[a]
rac-**14**		19.6	0.098
rac-**15**		11.5	0.205
rac-**16**		24.8	1.47
rac-**17**		2.77	0.124

[a] IC_{50} data are reported as the mean of at least three independent determinations, standard error of the mean ≤ 30%.

Reported 2-anilinobenzamides are indole analogues from which the best SIRT1 inhibitor **18** has also been tested in a fluorescence based assay for SIRT2 with an IC_{50} value of 74 μM for SIRT2 and 17 μM for SIRT1. Although the structures of **18** and **14** have structural similarities (the same distance between amine and amide groups), compound **14** is more rigid, and they have different aromatic ring systems. It has been reported that the structure activity relationships differ. The enzyme kinetic assay claimed that compound **18** exhibited a noncompetitive inhibition with NAD^+ but a competitive inhibition with the acetylated lysine substrate (Suzuki *et al.* 2006). Compound **14** has not been reported to have competitive inhibition with the acetylated lysine substrate (Napper *et al.* 2005). However, the analysis of the X-ray structure of SIRT2 and preliminary docking simulations using cambinol **8**, **12**, and **17** have suggested that compounds interact with the nicotinamide site C of SIRT2 (Neugebauer *et al.* 2008) and that they are the noncompetitive inhibitors with NAD^+ (Heltweg *et al.* 2006, Napper *et al.* 2005, Neugebauer *et al.* 2008).

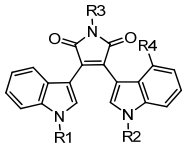


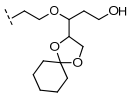
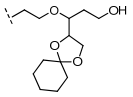
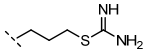
18

A systematic study for identification of lead structures for sirtuins from drugs that target enzymes or receptors that bind adenosine-containing cofactors or ligands has been conducted (Trapp *et al.* 2006). Bis(indolyl)maleimides were originally discovered as ATP-competitive protein kinase C inhibitors (table 2.5). The plain compound **19** without any substituent had an IC_{50} value of 4.7 µM for SIRT2. Compounds **20** and **21** having a bulky substituent on one of the indole nitrogen have slightly increased inhibitory activities. The IC_{50} values of **20** and **21** were 2.8 µM and 2.5 µM for SIRT2, respectively. Compounds **19**–**21** had roughly five-fold inhibitory activities against SIRT1. However, the best compound of the series, **22** had the IC_{50} value of 0.8 µM for SIRT2 and 3.5 µM for SIRT1, respectively (Trapp *et al.* 2006). The series of compounds was tested on a homogenous deacetylase assay using a fluorescent lysine derivative that was developed in the group of Heltweg *et al.* (2005). In addition, **22** was also tested in the scintillation assay, which gave the IC_{50} values of 1.1 µM and 5.1 µM for SIRT2 and SIRT1, respectively (Trapp *et al.* 2006).

Furthermore, **22** exhibited also an *in vivo* inhibitory activity as it induced hyperacetylation of tubulin tested on A549 human lung adenocarcinoma cells. Competition analysis for **22** suggested that the inhibition is competitive with NAD^+ and noncompetitive with the acetylated lysine substrate. In addition, the docking results suggested that **22** interacts with the adenine binding pocket (figure 2.2). The results of the competition and the docking analysis supported to each other (Trapp *et al.* 2006).

Table 2.5. Bis(indolyl)maleimides and their inhibitory activities for SIRT2 and SIRT1 (Trapp *et al.* 2006).



Compd	R1	R2	R3	R4	IC_{50} ± SE (μM)[a] SIRT2	Inhibition at 50 μM, % [a] SIRT1	IC_{50} ± SE (μM)[a] SIRT1
19	H	H	H	H	4.7 ± 1.1	52.7%	-
20		H	H	H	2.8 ± 1.2	77.5%	-
21		H	H	F	2.5 ± 0.6	71.9%	-
22		CH_3	H	H	0.8 ± 0.2	-	3.5 ± 0.4

[a] Values are means ± SE of duplicate experiments.

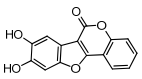
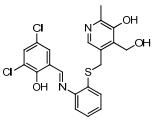
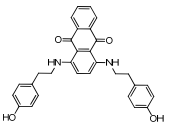
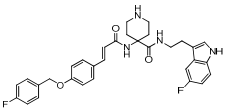
Compound **23** (A3) (table 2.6) was published at the same time with sirtinol **3** (table 2.2). It was found by a high throughput, phenotypic screening in cells by Grozinger *et al.* (2001). Compound **23** has been reported to have an IC_{50} value of 45 μM for SIRT2. The molecular structures of **23** and **3** are planar and aromatic, similar to the adenine and nicotinamide moieties of NAD^+. However, the imine derived from 2-hydroxy-1-naphthaldehyde in the structure of sirtinol **3** was claimed to be essential for the inhibitory activity and analogues of **23** have not been published thereafter (Grozinger *et al.* 2001).

Molecular modelling and virtual screening has been employed to find novel structural scaffolds for SIRT2 inhibitors. Compounds **24** and **25** are commercial compounds discovered from the Maybridge database (Maybridge Chemical Company Ltd) and tested using a radioactive [^{3}H]-substrate based assay (table 2.6). The determined IC_{50} values were 74.3 μM and 56.7 μM, respectively. Phenol groups of compounds **24** (CD 04097) and **25** (JFD 00244) were suggested to be capable of acting as hydrogen-bond

donors and, together with a hydrophobic moiety, to form an active SIRT2 pharmacophore (Tervo *et al.* 2004). According to Tervo *et al.* (2004), the naphthol moiety of sirtinol **3** can be replaced by a phenolic moiety without the loss of the inhibitory activity.

Successful virtual screening methods have also been used to discover structurally diverse inhibitors of SIRT2. One of the potent compounds ordered from LeadQuest Compound Library (Tripos Associates) **26** (Tripos 360702) had an indole moiety (table 2.6). Compound **26** had an IC_{50} value of 51 μM for SIRT2 (Tervo *et al.* 2006).

Table 2.6. Compounds **23**–**26** and their IC_{50} values for SIRT2.

Compd	Structure	IC_{50} (μM) SIRT2	Reference
23 (A3)		45 ± 3[a]	(Grozinger *et al.* 2001)
24 (CD 04097)		74.3 ± 1.5[a]	(Tervo *et al.* 2004)
25 (JFD 00244)		56.7 ± 4.2[a]	(Tervo *et al.* 2004)
26 (Tripos 360702)		51 (27-75)[b]	(Tervo *et al.* 2006)

[a] Average and standard deviation values were obtained from the IC_{50} determination performed in triplicate. [b] 95% confidence intervals given in parentheses.

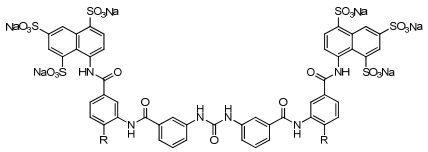
Suramin **27**, originally used for the treatment of trypanosomiasis and onchocerciasis, is a symmetric polyanionic naphthylurea, which was first reported to inhibit SIRT1 (Howitz *et al.* 2003). The approach of previously identified adenosine mimics from the same group (Trapp *et al.* 2006) had led to assume a similar structure-activity relationship for suramin than for the bis(indolyl)maleimides (table 2.5). However, it has been reported that suramin interacts with the nicotinamide binding site, the C site of

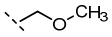
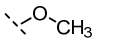
SIRT2 and, hence, would function as SIRT inhibitor. The binding of suramin has been claimed to be noncompetitive (Trapp *et al.* 2007).

Suramin and several of its analogues were tested in a fluorescence based assay using ZMAL as the acetylated peptide substrate (table 2.7) (Heltweg and Jung 2003). The IC_{50} values of suramin **27** were 1.150 μM and 0.297 μM for SIRT2 and SIRT1, respectively. The best suramin analogue for SIRT2 was **34**, where the methyl substituents on the benzene rings of suramin had been replaced by chlorine atoms. This compound had an IC_{50} value of 0.407 μM. Thus the best suramin analogue for SIRT1 was **28**, where the methyl substituents on the benzene rings of suramin had been replaced by hydrogen atoms. This compound had IC_{50} values of 0.585 μM and 0.165 μM for SIRT2 and SIRT1, respectively. Compounds **29**, **30**, **31**, and **35** with ethyl, isopropyl, fluoro, and methoxy groups as replacements of the methyl substituents on the benzene rings had similar inhibitory activities for SIRT2 than **28** ranging from 0.449 μM to 0.612 μM. The same compounds **29**–**31** and **35** had slightly better IC_{50} values for SIRT1, between 0.223 μM and 0.308 μM. Compounds **32** and **33** with larger substituents at these positions were reported to have a decreased inhibitory activity compared to suramin **27** for SIRT1. Compound **32** had slightly better IC_{50} value for SIRT2 than suramin **27** (Trapp *et al.* 2007).

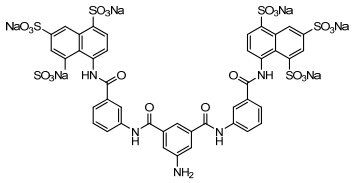
In addition, the structure of compounds **28**–**35** was simplified by replacing the central symmetric bis(*meta*-carboxyphenyl)urea moiety by an isophthalic acid. The central benzene ring was substituted with an amino group resulting in **36**. This is one of the most potent SIRT1 inhibitors published. The inhibitory activity of **36** was about 24-times lower for SIRT2. The IC_{50} values of **36** were 2.261 μM 0.093 μM for SIRT2 and SIRT1, respectively. Moreover, compound **37**, which was a truncated compound **31** (one half of it) had IC_{50} values of 15.534 μM and 0.525 μM for SIRT2 and SIRT1, respectively (Trapp *et al.* 2007).

Table 2.7. Suramin and its analogues and their inhibitory activities for SIRT2 and SIRT1 (Trapp *et al.* 2007).

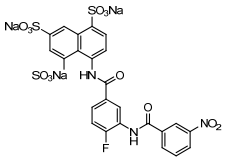


Compd	R	IC_{50} (μM) ± SE[a] SIRT2	IC_{50} (μM) ± SE[a] SIRT1
27 suramin	CH_3	1.150 ± 0.123	0.297 ± 0.010
28	H	0.585 ± 0.053	0.165 ± 0.019
29		0.612 ± 0.124	0.223 ± 0.014
30		0.449 ± 0.025	0.308 ± 0.009
31	F	0.467 ± 0.175	0.283 ± 0.012
32		0.929 ± 0.055	1.713 ± 0.131
33		1.725 ± 0.140	0.662 ± 0.028
34	Cl	0.407 ± 0.099	0.339 ± 0.011
35		0.510 ± 0.031	0.233 ± 0.012

[a] Values are means ± SE of duplicate experiments.



36 Substituted *meta*-anthranilic acid derivative

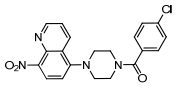
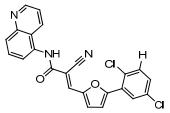
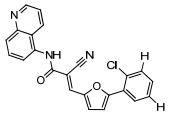
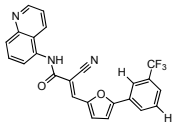
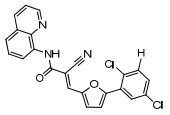


37

The molecular weight of the most potent SIRT2 inhibitor **34** of the series is 1491.5 g/mol. It has been reported that the cellular uptake and the bioavailability of the compounds are generally limited considering the high molecular weight of the compounds and highly polar sulfonic acids in the chemical structures (Trapp *et al.* 2007).

Compound **38** has been reported to be a SIRT2 inhibitor both *in vitro* and *in vivo* (table 2.8) (Outeiro *et al.* 2007). Compound **39** (AGK2) was revealed from the library search of designed 200 structural analogues of **38**. Compound **39** inhibited SIRT2 with an IC_{50} value of 3.5 μM and SIRT1 with an IC_{50} value of over 50 μM tested *in vitro* in a fluorescence based assay. *N*-(5-Quinolyl)propenamide **39** has been reported to link SIRT2 inhibition and neurodegeration. The cellular inhibition by **39** rescued α-synuclein toxicity and protected against dopaminergic cell death, both biological changes caused by Parkinson disease (Outeiro *et al.* 2007).

Table 2.8. AGK lead structures and their IC_{50} values for SIRT2 and SIRT1 (Outeiro *et al.* 2007).

Compd	Structure	IC_{50} (μM) SIRT2[a]	IC_{50} (μM) SIRT1[a]
38		35	> 50
39 (AGK2)		3.5	> 50
40		5.5	> 50
41		6	> 50
42		> 50	> 50

[a] The standard error not reported.

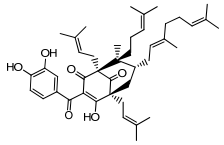
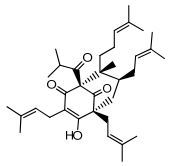
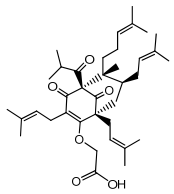
Compound **40** with one chloro-substituent and compound **41** with a trifluoromethyl-substituent at the 3-position gave the IC_{50} values 5.5 μM and 6 μM for SIRT2, respectively. As a reference, *N*-(2-quinolyl)propenamide **42** gave an over 50 μM

inhibitory activity for SIRT2. Compound **39** has been claimed to interact with the C site of SIRT2 by mimicking nicotinamide. So far, the mechanism for the effect of SIRT2 inhibition remains unclear. However, microtubule stabilization itself resulting from SIRT2 inhibition could be an important factor in neuroprotection (Outeiro *et al.* 2007).

In the fluorescence based assay at University of Kuopio compound **39** gave IC_{50} values of 42.5 μM and 63.2 μM for SIRT2 and SIRT1, respectively (unpublished results).

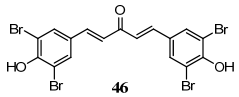
Also a few natural products have been tested as sirtuin inhibitors (Gey *et al.* 2007). Guttiferone G **43**, hyperforin **44** and **45** (a synthetic derivative of **44**) have been tested in a radioactive [^{3}H]-substrate based assay for SIRT2 and SIRT1 (table 2.9). Aristoforin **45** was the most potent compound of the series with IC_{50} values of 21 μM and 7 μM for SIRT2 and SIRT1, respectively. However, guttiferone **43** was almost equipotent. The IC_{50} values were slightly better for SIRT1 than for SIRT2. The cytotoxicity of **43**–**45** and the effect on cell proliferation were also tested. It has been reported that **43** and **45** were less toxic than **44** and they were stronger inhibitors of the cell proliferation. The results indicated the value of the phloroglucinol scaffold for the design of SIRT2 and SIRT1 inhibitors (Gey *et al.* 2007).

Table 2.9. Phloroglucinol derivatives and their IC_{50} values for SIRT2 and SIRT1.

Compd	Name	Structure	IC_{50} (μM) SIRT2	IC_{50} (μM) SIRT1
43	(+)-guttiferone G		22 ± 0.5	9 ± 0.2
44	hyperforin		28 ± 0.2	15 ± 0.5
45	aristoforin		21 ± 1	7 ± 0.2

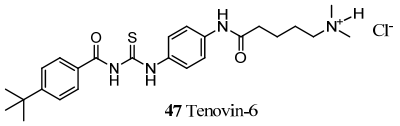
There are several post-transitional modifications of core histones, from which lysine acetylation and deacetylation are just one that lead gene expression or silencing to fulfil the requirements of the cell (Nightingale *et al.* 2006). These modifications in gene expression are also called epigenetics and they form a complex network where the effect of one modification will most probably change next one. There are certain diseases, such as cancer and central nervous system disorders, which take advantage of the complex network of cellular pathways and signals (Biel *et al.* 2005, Egger *et al.* 2004). A multitarget-directed drug design strategy has been proposed which aim at the design of a single compound for several targets in a disease is to enhance an efficacy and to improve a safety of a therapy. The series of epigenetic multiple ligands have also been tested for SIRT2 and SIRT1. Compound **46** has been reported to inhibit SIRT2 activity 100% at 25 μM and SIRT1 activity 61% at 25 μM tested in the fluorescence based assay (Mai *et al.* 2008). Compound **46** is symmetric and it has the 4-hydroxyphenyl-propenoyl structure, one or both qualities which are reported also to be

found from several other SIRT2 inhibitors (Tervo *et al.* 2004, Tervo *et al.* 2006, Trapp *et al.* 2006, Trapp *et al.* 2007).



46

Small active compounds for SIRT2 and SIRT1 have also been discovered using a mammalian cell-based screening assay (Lain *et al.* 2008). The advantage of the assay is that potent compounds that are found are already acceptable for further experiments (not toxic to cells). Compounds called tenovins have recently been found by cell-based screening and they have been reported to inhibit SIRT2 and SIRT1. The IC_{50} values of tenovin-6 **47** are 10 μM and 21 μM for SIRT2 and SIRT1, respectively, tested *in vitro* in a fluorescence based assay. In addition, tenovins-1 and -6 have also been claimed to delay growth of tumors without general toxicity. Hence, Lain *et al.* (2008) suggested that tenovins should be considered as valuable leads in medicinal chemistry.

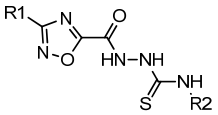


47 Tenovin-6

Oxadiazole-carbonylaminothiourea has been reported to be a potent structural scaffold for SIRT2 and SIRT1 inhibitors. It has been found by virtual database screening tested *in vitro* in microplate filtration based assay (Huhtiniemi *et al.* 2008). The assay was based on the release of a radioactively labelled nicotinamide from NAD^+ and its detection by thin layer chromatography (McDonagh *et al.* 2005, Tanny and Moazed 2001). Compound **48** was the reported hit compound and a series of analogues were synthesized and tested for SIRT2 and SIRT1 (table 2.10). The inhibitory activity of **48** was 57 μM and 192 μM for SIRT2 and SIRT1, respectively. The inhibitory activity of SIRT2 could not be improved. However, compound **50** was almost equipotent with an IC_{50} value of 74 μM for SIRT2. Compound **49** was the most potent SIRT1 inhibitor with an IC_{50} value of 13 μM. The inhibitory activity of **49** for SIRT2 was almost nine-times lower with a IC_{50} value of 113 μM (Huhtiniemi *et al.* 2008).

Huhtiniemi *et al.* (2006) have published a comparative model of SIRT1 and studied the binding modes of compound **14**. Compound **48** was found by virtual database screening of novel inhibitors which share a similar binding site with **14** (table 2.4). This binding site is also known as the C site. Thus it has been reported that the inhibition mechanism of oxadiazole-carbonylaminothioureas is most likely the preventation of the productive conformation of NAD^+ (Huhtiniemi *et al.* 2008).

Table 2.10. Oxadiazole-carbonylaminothioureas and their IC_{50} values SIRT2 and SIRT1 (Huhtiniemi *et al.* 2008).



Compd	R1	R2	IC_{50} (μM) SIRT2[a]	IC_{50} (μM) SIRT1[a]
48	4-*t*-Bu-Ph	3-CF_3-Ph	57 (26-125)	192 (104-354)
49	1-naphthyl	3-CF_3-Ph	113 (64-200)	13 (5-37)
50	1-naphthyl	4-CF_3-Ph	74 (47-115)	318 (140-723)

[a] 95% confidence intervals for IC_{50} given in parentheses.

2.8.2 Substrate-based inhibitors

Substrate analogues have also been used as SIRT2 inhibitors. Initially, the N^{ε}-thioacetylated lysine was considered as a functional mimic for the *N*-acetylated lysine. However, the N^{ε}-thioacetylated Lys382 of human p53 sequence (amino acid residue 372-389) **51** inhibited the deacetylation reaction (Fatkins *et al.* 2006). The peptide **51** had IC_{50} values of 1.8 μM and 1.7 μM for SIRT2 and SIRT1, respectively (table 2.11) (Fatkins and Zheng 2008). On the other hand, N^{α}-Fmoc-N^{ε}-thioacetyl-lysine **52** and N^{α}-acetyl-N^{ε}-thioacetyl-lysine **53** were only inhibiting SIRT1 at high concentrations (Fatkins *et al.* 2006).

The research was continued with the SIRT2 substrate human α-tubulin sequence 36-44 **54** and the SIRT3 substrate human acetyl-coenzyme A synthetase 2 (AceCS2) sequence 633-652 **55** (Fatkins and Zheng 2008). The sequence of α-tubulin had an IC_{50} value of 11.4 μM and had a weak selectivity for SIRT2 over SIRT1 and SIRT3. The

sequence from AceCS2 **55** had an IC_{50} value of 0.9 μM for SIRT1 and it had a weak selectivity for SIRT1 over SIRT2 and SIRT3 (table 2.11) (Fatkins and Zheng 2008).

Several pentapeptides, which had an N^{ε}-acetylated lysine in the middle of the sequence, were identified from a combinatorial peptide library to mimic SIRT1 substrates (Garske and Denu 2006). Due to the results, the short peptide **56** based on human p53 sequence has been determined to have about six-times lower inhibitory activity than the longer peptide **51** (table 2.11) (Fatkins and Zheng 2008). The HPLC based assay was used to determine the inhibitory activities of the peptides **51**–**56** (Fatkins *et al.* 2006).

These results have given a starting point for mechanism-based inhibitors of Sir2 deacetylases. The reported mechanism of N^{ε}-thioacetylated lysine of human histone H3 sequence (11 amino acid residues) **57** has indicated that the dethioacetylation reaction proceeds with the same mechanism as the deacetylation reaction, the only difference is that it is extremely slow. The dethioacetylation reaction has been claimed to stall at the 1′-*S*-alkylamidate intermediate after nicotinamide formation. As a reference, N^{ε}-trifluoroacetyl-lysine peptide was reported to exhibit a competitive inhibition with the acetyl lysine substrate in contrary to **57**. The IC_{50} value of **57** was reported to be 5.6 μM and 2.0 μM for SIRT2 and SIRT1, respectively (table 2.11) (Smith and Denu 2007b).

Binding studies of several acetyl-lysine analog peptides have suggested that the hydrophobicity of the analogs has better correlation to binding than the electronic and steric effects. The propionyl-lysine peptide was found to bind tighter to Hst2 compared with the acetyl-lysine peptide. The depropionylation reaction was measurable suggesting that the propionyl-lysine proteins may function as sirtuin substrates *in vivo*. The result was suggested to be applicable towards the development of substrate-based inhibitors (Smith and Denu 2007a).

Table 2.11. Substrate-like inhibitors and their IC_{50} values for SIRT2 and SIRT1.

Compd	Source	Sequence	IC_{50} (μM) SIRT2	IC_{50} (μM) SIRT1	IC_{50} (μM) SIRT3	Reference
51	human p53 (372-389)	H_2N-KKGQSTSRHK**K**(N^{ε}-ThioAc)LMFKTEG-OH	1.8 ± 0.3	1.7 ± 0.4	67.3 ± 2.4	(Fatkins and Zheng 2008)
52	-	N^{α}-Fmoc-N^{ε}-thioacetyl-lysine	ND [a]	2000 (IC_{25})	ND [a]	(Fatkins *et al.* 2006)
53	-	N^{α}-Acetyl-N^{ε}-thioacetyl-lysine	ND [a]	NI [b] at 2 mM	ND [a]	(Fatkins *et al.* 2006)
54	human α-tubulin (36-44)	H_2N-MPSD**K**(N^{ε}-ThioAc)TIGG-OH	11.4 ± 1.1	116.8 ± 12.0	449.4 ± 18.4	(Fatkins and Zheng 2008)
55	human AceCS2 (633-652)	H_2N-KRLPKTRSG**K**(N^{ε}-ThioAc)VMRRLLRKII-OH	4.3 ± 0.3	0.9 ± 0.2	4.5 ± 2.0	(Fatkins and Zheng 2008)
56	human p53 (380-384)	H_2N-HK**K**(N^{ε}-ThioAc)LM-OH	ND [a]	About 10 [c]	ND [a]	(Fatkins and Zheng 2008)
57	human histone H3	H_2N-KSTGG**K**(N^{ε}-ThioAc)APRKQ-OH	5.6 ± 0.8	2.0 ± 0.2	2.3 ± 0.3	(Smith and Denu 2007b)

[a] Not determined. [b] No inhibition. [c] The exact value was not reported.

2.9 References

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3 AIMS OF THE STUDY

The aim of the study was to design and synthesize new SIRT2 inhibitors, which would be suitable for further studies of the biological function of SIRT2. The more specific aims were:

1. To develop new SIRT2 inhibiting compounds with improved inhibitory activity, increased solubility and decreased toxicity as compared to the earlier known SIRT2 inhibitors.

2. To search for new molecular backbones for SIRT2 inhibitors based on the results from molecular modelling and virtual screening.

3. To study the structure-activity relationships by different replacements on the new SIRT2 inhibitors in collaboration with molecular modellers.

4. To develop new SIRT2 inhibitors based on the peptide sequences of known SIRT2 substrates.

5. To study the selectivity (between SIRT1 and SIRT2) of the new inhibitors in order to determine possible differences in their binding interactions.

4 GENERAL EXPERIMENTAL PROCEDURES

4.1 General synthetic procedures

This chapter describes the general synthetic procedures in this thesis. Detailed synthetic procedures are given in Chapters 5-8.

All reagents and solvents obtained from commercial suppliers were used without further purification. When needed, the solvents were dried over 3 Å or 4 Å molecular sieves. Anhydrous tetrahydrofuran (THF) was prepared by drying over sodium followed by distillation under argon. The glassware for anhydrous conditions was dried at 140 ^{o}C and cooled in a desicator or under argon and the reactions were performed under argon or nitrogen. The light sensitive reactions were protected by aluminium foil.

When possible, the described reactions and work-up were first monitored by thin layer chromatography using aluminum sheets precoated with Merck silica gel 60 F_{254}. Samples were visualized by UV light, heating, or staining using potassium permanganate or ninhydrine in combination with heating. Purifications by flash chromatography were performed on J. T. Bakers silica gel for chromatography (pore size 60 Å, particle size 50 mm), or by CombiFlash® chromatography on a commercial silica column, or by Chromatotron® on silica gel plates made of Merck silica gel 60 PF_{254}-containing gypsum (particle size 5-40 μM).

Peptides were synthesized on a solid phase manually or on a peptide synthesizer an Apex 396 DC multiple peptide synthesizer (Advanced ChemTech, Louisville, KY).

4.2 Analytical procedures

Nuclear magnetic resonance (NMR) spectroscopy. ^{1}H and decoupled ^{13}C NMR spectra were recorded on a Bruker Avance 500 spectrometer (500.1 MHz for ^{1}H and 125.8 MHz for ^{13}C, respectively) at 25 ^{o}C. The FID files were processed with TopSpin software (version 2.1 or older, Bruker Biospin) to obtain NMR spectra. The chemical shifts (δ) are expressed in ppm relative to tetrametylsilane (δ 0.00) as an internal standard.

The *N*-amide bond of proline has energetically similar *cis* and *trans* isomers (rotamers). These rotamers have slightly different chemical shifts as described in Chapter 7. The minor rotamer has an integral corresponding to 30% of the integral of the major rotamer.

The chemical shifts of the proton resonances (^{1}H and COSY) for the described peptides in Chapter 8 are presented on tables and were determined at 500.1 MHz in D_2O with DMSO-d_6 (δ 2.71) as an internal standard or in DMSO-d_6 with tetrametylsilane (δ 0.00) as an internal standard. ^{13}C- and attached proton test (APT)-spectra were determined at 125.8 MHz in D_2O with DMSO-d_6 (δ 39.39) as an internal standard or in DMSO-d_6 with $CDCl_3$ (δ 79.16) as an internal standard.

Electrospray ionization mass spectroscopy (ESI-MS). Electronspray ionization mass spectra were acquired by an LCQ quadrupole ion trap (Finnigan MAT, San Jose, CA), or LTQ linear ion trap (Thermo Fisher Scientific), or 6410 Triple Quad MS (Agilent Technologies) mass spectrometers with an electron spray ionization source.

Elemental analysis. Elemental analyses were carried out on a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer.

High pressure liquid chromatography (HPLC) purification. Peptides described in Chapter 8 were purified by preparative HPLC (Shimadzu LC-10A*vp* (Fennolab, Fenno Medical Oy)) on a reverse-phase C18 column (Supelcogel ODP-50, 25 cm * 21.2 mm, 5 μm) with a linear gradient of 5-90% solvent B (0.05% acetic acid/ acetonitrile) in solvent A (0.05% acetic acid/ H_2O) in 30 min with the flow rate 10mL/min. The peptide was detected by UV at λ = 215 nm. The purity of each peptide was verified by NMR.

4.3 Expression of human SIRT1 and SIRT2 recombinant proteins

Human SIRT1 cDNA (as in RefSeq NM_012238) and SIRT2 cDNA (transcript variant 1 as in RefSeq NM_012237) were amplified by polymerase chain reaction (PCR) with the Advantage cDNA Polymerase Mix (Takara/Clontech for SIRT1 and BD/Clontech for SIRT2) using a sense primer 5'-GCGGATCCAAGATGGCGGACGAGG and an antisense primer 5'-GAACTATCCATCAAACAAATCATAG for SIRT1 and a sense primer 5′-CCGGATCCATGGCAGAGCCAGAC and an antisense primer 5′-CAGAATTCACTGGGGTTTCTCCC for SIRT2. The primers contained recognition sites for *Bam*HI and *Eco*RI (only SIRT2) restriction endonucleases, respectively (underlined). cDNA synthesized on total RNA from the WI-38 normal human lung fibroblast cell line (ATCC) by M-MLV Reverse Transcriptase (Promega) was used as a template for PCR. The PCR fragment was digested with the appropriate restriction

endonucleases and cloned into the *Bam*HI-SmaI (SIRT1) or the *Bam*HI-*Eco*RI (SIRT2) digested pGEX-2T bacterial expression vector (GE Healthcare or Amersham Biosciences) under the control of a Ptaq inducible promoter. The identity of inserts in the resulting plasmid pTe34 (pGEX2T-SIRT1) and pTe25 (pGEX2T-SIRT2) were verified by sequencing. The cultures of *E. coli* strain DH5α bearing either pTe34 or pTe25 were induced by 0.2 mM isopropyl-β-D-1-thiogalactopyranoside at 23°C for 4-6 h in terrific broth rich medium with vigorous shaking in baffled Erlenmeyer flasks, and the soluble overexpressed recombinant proteins were affinity purified on Glutatione Sepharose 4B medium or GSTrapp FF colums (GE Healthcare or Amersham Biosciences). The size and the identity of the proteins obtained were further verified by SDS-PAGE analysis and protein mass spectrometry, respectively. The purified recombinant GST-SIRT1 and GST-SIRT2 fusion proteins showed robust protein deacetylase activity. Their activity was dependent on NAD^+ and could be inhibited by nicotinamide, both of which are characteristic features attributed to mammalian SIRT1 and SIRT2. In contrast, the control GST protein preparations obtained from unmodified pGEX-2T vector showed no deacetylase activity. The purified recombinant GST-SIRT1 and GST-SIRT2 fusion proteins were stored at -80^{o}C in 20% glycerol.

4.4 *In vitro* assays for SIRT1 and SIRT2 activities

The SIRT2 inhibitory activity of the synthesized compounds described in Chapter 5 were tested on *in vitro* radioactive [^{3}H]-substrate based deacetylation assay according to the histone deacetylase assay kit protocol (Upstate Biotechnology). Whereas, the SIRT1 and SIRT2 inhibitory activities of the synthesized compounds described in Chapter 6-8 were tested on *in vitro* Fluor de Lys assay modified from the BioMol product sheet. The proper test system for the inhibitory activity measurements was chosen depending on the properties of the compounds. The optic properties of compounds described in Chapter 5 disturbed the use of the fluorescence based assay.

Radioactive [^{3}H]-substrate based deacetylation assay. The α-tubulin peptide MPSDKTIGG was chemically acetylated with [^{3}H]-acetic acid and a benzotriazol-1-yloxytris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) reagent. The labeled peptide was purified with HPLC on C18 reverse phase column. The SIRT2

deacetylation reaction was performed in 100 µl of HDAC buffer (15 mM Tris/HCl, pH 7.9, 0.25 mM EDTA, 10 mM NaCl, 10% glycerol (v/v), 10 mM mercaptoethanol; Upstate Biotechnology) with 40 000 cpm [^{3}H]-peptide substrate and 500 µM NAD^+ (Sigma) as cosubstrate. The SIRT2 inhibitors were diluted in DMSO. The concentration of DMSO was always kept equal in every test tube at 5%. The reaction was started by adding 1 µg of recombinant GST-SIRT2 and incubated at 37^{o}C overnight. The released [^{3}H]-acetyl product was extracted with ethyl acetate and quantified with a liquid scintillation counter (Wallac WinSpectral 1414 or Wallac 1450 MicroBeta). Radioactive [^{3}H]-substrate based deacetylation assay has been tested beforehand to be linear up to 12 h. Each experiment was repeated at least three times. The IC_{50} values were based on a 6-point dose-response determination (200 µM, 100 µM, 50 µM, 25 µM, 10 µM, and 0 µM) where more necessary dose points were added between the critical concentrations depending on the inhibitor and calculated using Origin graphic program version 6.0 (MicroCal Software, Inc.).

Fluor de Lys fluorescence based assay. Fluor de Lys-SIRT1 peptide substrate is based on residues 379-382 of p53 (Arg-His-Lys-Lys(Ac)) and Fluor de Lys-SIRT2 peptide substrate is based on residues 317-320 of p53 (Gln-Pro-Lys-Lys(Ac)) (BioMol KI-177 for SIRT1 and KI-179 for SIRT2). The assays were carried out using Fluor de Lys-acetylated peptide substrate (50 µM for SIRT1 and 125 µM for SIRT2), 500 µM NAD^+ (N6522, Sigma), recombinant GST-SIRT1/2-enzyme and SIRT1/2-assay buffer (HDAC assay buffer, KI-143, supplemented with 1 mg/ml BSA, A3803, Sigma). The buffer, SIRT1/2-enzyme, NAD^+ and DMSO/compounds in DMSO (2.5 µl in 50 µl total volume of reaction mixture; DMSO from Sigma, D2650) for testing were preincubated for 5 min at room temperature (rt). The reaction was started by adding the substrate, and the reaction mixture was incubated for 1 h (SIRT1) or 3 h (SIRT2) at 37°C. After that Fluor de Lys-developer (KI-176) plus 2 mM nicotinamide in 50 µl were added and incubation was continued for 45 min at 37°C. Fluorescence readings were obtained using the Victor^{TM} 1420 Multilabel Counter (Wallac, Finland) with excitation wavelength 360 nm and emission 460 nm. The fluorescent signal indicated deacetylation of Lys382 (SIRT1) or Lys320 (SIRT2).

The IC_{50} values of the fluorescence based assay were based on a 8-point dose-response determination (1000 μM, 100 μM, 10 μM, 1 μM, 0.1 μM, 0.01 μM, 0.001 μM, and 0.0001 μM) where more necessary dose points were added between the critical concentrations depending on the inhibitor. Each experiment was repeated at least three times and calculated using GraphPad Prism Software version 4.03 (© 1992-2005 GraphPad Software, Inc.).

5 *N,N'*-BISBENZYLIDENEBENZENE-1,4-DIAMINES AND *N,N'*-BISBENZYLIDENENAPHTHALENE-1,4-DIAMINES AS SIRT2 INHIBITORS*

Abstract: A series of *N,N'*-bisbenzylidenebenzene-1,4-diamine and *N,N'*-bisbenzylidenenaphthalene-1,4-diamine derivatives were synthesized as inhibitors for SIRT2. The design of the new compounds was based on two earlier reported hits from molecular modelling and virtual screening. The most potent compound was *N,N'*-bis(2-hydroxybenzylidene)benzene-1,4-diamine, which was equipotent with the most potent hit compound and well-known SIRT2 inhibitor sirtinol.

* Adapted with permission from: Kiviranta PH, Leppänen J, Kyrylenko S, Salo HS, Lahtela-Kakkonen M, Tervo AJ, Wittekindt C, Suuronen T, Kuusisto E, Järvinen T, Salminen A, Poso A, Wallén EAA. *N,N'*-Bisbenzylidenebenzene-1,4-diamines and *N,N'*-Bisbenzylidenenaphthalene-1,4-diamines as Sirtuin Type 2 (SIRT2) Inhibitors. Journal of Medicinal Chemistry 49: 7907-11, 2006.

5.1 Introduction

The starting point for the design and synthesis of the novel SIRT2 inhibitors described in this chapter was the Maybridge database search done by Tervo *et al.* (2004). The crystal structure of SIRT2 was utilized in molecular modelling and virtual screening (Finnin *et al.* 2001). A search in the Maybridge database resulted in five compounds that showed inhibitory activity for SIRT2. The IC_{50} values of the two most potent compounds 1,4-bis[2-(4-hydroxyphenyl)ethylamino]anthraquinone **1** (Maybridge JFD00244) and 5-(2-{[1-(3,5-dichloro-2-hydroxyphenyl)meth-(*E*)-ylidene]amino} phenylsulfanylmethyl)-4-hydroxymethyl-2-methylpyridin-3-ol **2** (Maybridge CD04097) were 56.7 and 74.3 µM, respectively (figure 5.1) (Tervo *et al.* 2004).

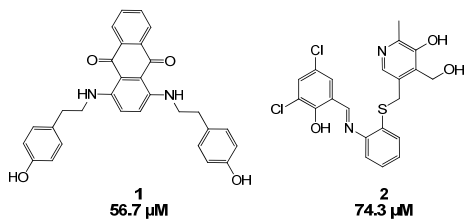


Figure 5.1. Two most potent compounds found by molecular modelling and virtual screening (Tervo *et al.* 2004).

The two compounds in figure 5.1 have structural backbones that are new for SIRT2 inhibitors. The common structural features in the two compounds in figure 5.1 are that both have a central aromatic group, which is disubstituted with two tethered hydroxy substituted aromatic groups. The phenolic hydroxyl groups of sirtinol and its analogues have been reported to be important for inhibitory activity (Grozinger *et al.* 2001, Mai *et al.* 2005). The symmetrical **1** is 1,4-disubstituted. The length of the tether is three atoms, and the tether is connected via a nitrogen atom with an amine function. The unsymmetrical **2** is 1,2-disubstituted. The lengths of the tethers are two atoms, and the tethers are connected via a nitrogen atom with an imine function or via a sulfur atom with a sulfide function.

A new series of SIRT2 inhibitors with the general structure in figure 5.2 was designed based on **1** and **2**. In the new series of compounds the central aromatic group is a 1,4-disubstituted benzene or naphthalene, the tether length is two atoms, it is connected via

a nitrogen atom with an amine or an imine function, and the tethered aromatic group is a phenyl group. The choice of a naphthalene group as the central aromatic group was also supported by the fact that naphthalene derivatives have been reported to inhibit yeast Sir2 inhibitors (Posakony *et al.* 2004).

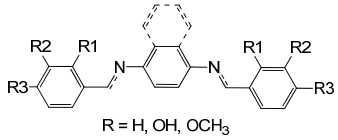
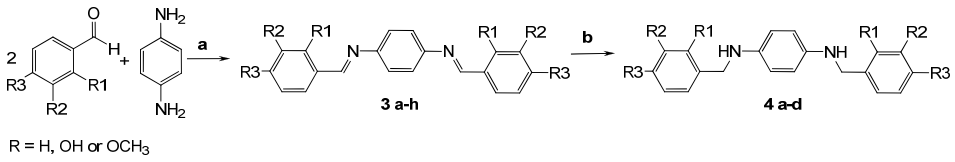


Figure 5.2. General structures of the new series of compounds.

5.2 Synthetic chemistry

The compounds were synthesized via imine formation from benzene-1,4-diamine (or naphthalene-1,4-diamine) and the appropriate aldehyde (scheme 5.1). The imine groups of the compounds from benzene-1,4-diamine were reduced with sodium borohydride.



Scheme 5.1. Synthetic route for **3** and **4**. Reagents: (a) EtOH; (b) $NaBH_4$, 1,2-dimethoxyethane.

5.3 Results and discussion

The structures of the compounds and their SIRT2 inhibitory activities at 200 μM are presented in tables 5.1, 5.2, and 5.3, respectively. The IC_{50} values are presented for the most potent compounds in table 5.4. The unsubstituted *N*,*N*'-bisbenzylidenebenzene-1,4-diamine (**3a**) gave an inhibition of 35% at 200 μM (table 5.1). This shows that the new backbone is a moderate inhibitor of SIRT2, even when it is unsubstituted. *N*,*N*'-Bis(2-hydroxybenzylidene)benzene-1,4-diamine (**3b**) was the most potent inhibitor in the series with hydroxy substituted derivatives **3b**–**e**. Compound **3b** had an inhibition of 56% at 200 μM and an IC_{50} of 58.4 μM (table 5.4). The methoxy substituted derivatives

3f–**g** had lower inhibitory activities than the unsubstituted compound **3a**. The inhibitory activity of the *para*-substituted methoxy derivative **3h** was not determined because of an extremely low solubility.

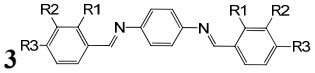
The reduction of the imine function of **3a**–**d** resulted in **4a**–**d** (table 5.2). Compounds **4a**–**d** are more flexible than **3a**–**d** because of deletion of the conjugated double bond between the aromatic rings. Compounds **4a**–**d** had a slightly lower inhibitory activity compared to compounds **3a**–**d**, the *ortho*-hydroxy substituted **4b** being again the most potent with an inhibition of 27% at 200 μM.

The unsubstituted *N*,*N*'-bisbenzylidenenaphthalene-1,4-diamine (**5a**) resulted in an inhibition of 18% at 200 μM (table 5.3). This showed that the replacement of the central benzene group by a naphthalene group lowered the inhibitory activity slightly. However, all hydroxy substituted derivatives **5b**–**d** show improved inhibitory activities compared to the unsubstituted **5a**. Interestingly, the *meta*- and *para*-substituted **5c** and **5d** are the most potent compounds in this series with inhibitions of 53% and 64% at 200 μM, respectively, and IC_{50} of 195.9 and 137.4 μM, respectively. The methoxy substituted **5e**–**g** are equipotent with the unsubstituted **5a**, but slightly less potent than the most potent hydroxy substituted **5c**–**d**.

When the compounds from tables 5.1, 5.2, and 5.3 are compared, it can be seen that the most potent compounds **3b**, **5c**, and **5d** have almost equal percentages of inhibition at 200 μM (56%, 53%, and 64%, respectively). Since these are only three compounds that have over 50% inhibition at 200 μM, their IC_{50} values were determined. In the *in vitro* assay for SIRT2 activity, poor solubility of several compounds was observed when determining the inhibition at higher concentrations. This was an additional reason not to determine the IC_{50} of compounds with less than 50% inhibition at 200 μM. The most potent compound **3b** has an IC_{50} of 58.4 μM which is equipotent with the most potent hit compound **1** and sirtinol (table 5.4). *N*,*N*'-Bis(3-hydroxybenzylidene)naphthalene-1,4-diamine (**5c**) and *N*,*N*'-bis(4-hydroxybenzylidene)naphthalene-1,4-diamine (**5d**) gave slightly higher IC_{50} values of 195.9 μM and 137.4 μM, respectively. The difference can be explained by the relatively large standard deviation of the percent inhibition of **3b** (56% ± 8.2%) at 200 μM. The Hill slopes of the inhibition curves did not deviate significantly from unity.

An interesting observation regarding the conformation of the compounds is that the most potent compound **3b** has hydroxyl groups in the *ortho*-positions, which create strong intramolecular hydrogen bonds with the electronegative nitrogen atoms. The hydrogen bonds have an effect on the NMR spectra of **3b**, giving a more downfield chemical shift of the hydroxyl groups (in DMSO-d_6). The same chemical shift cannot be observed for *N*,*N*′-bis(2-hydroxybenzyl)benzene-1,4-diamine (**4b**) but can again be observed for *N*,*N*′-bis(2-hydroxybenzylidene)naphthalene-1,4-diamine (**5b**).

Table 5.1. Structures of **3** and their % inhibition at 200 μM.

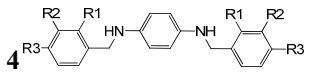


3

Compd	R1	R2	R3	Inhibition at 200 μM ± SD,[a] %
3a	H	H	H	35 ± 1.4
3b	OH	H	H	56 ± 8.2
3c	H	OH	H	12 ± 0.9
3d	H	H	OH	22 ± 1.6
3e	H	OH	OH	13 ± 0.2
3f	OCH_3	H	H	13 ± 0.5
3g	H	OCH_3	H	20 ± 1.2
3h	H	H	OCH_3	ND [b]

[a] SD = standard deviation. [b] The product did not dissolve.

Table 5.2. Structures of **4** and their % inhibition at 200 μM.

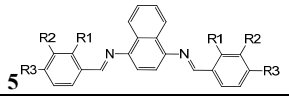


4

Compd	R1	R2	R3	Inhibition at 200 μM ± SD,[a] %
4a	H	H	H	21 ± 1.7
4b	OH	H	H	27 ± 2.4
4c	H	OH	H	8 ± 0.4
4d	H	H	OH	14 ± 0.5

[a] SD = standard deviation.

Table 5.3. Structures of **5** and their % inhibition at 200 µM.



5

Compd	R1	R2	R3	Inhibition at 200 µM ± SD,[a] %
5a	H	H	H	18 ± 0.5
5b	OH	H	H	38 ± 1.2
5c	H	OH	H	53 ± 1.9
5d	H	H	OH	64 ± 1.9
5e	OCH_3	H	H	34 ± 1.2
5f	H	OCH_3	H	20 ± 0.2
5g	H	H	OCH_3	17 ± 1.1

[a] SD = standard deviation.

Table 5.4. IC_{50} values of the most potent compounds.

Compd	IC_{50} ± SD,[a] µmol/L
sirtinol	45.1 ± 1.6
1	56.7 ± 4.2
2	74.3 ± 1.5
3b	58.4 ± 14.8
5c	195.9 ± 10.9
5d	137.4 ± 8.8

[a] SD = standard deviation

To elucidate the binding possibilities of the SIRT2 inhibitors, compounds **3b**, **5c**, **5d**, sirtinol, **1**, and **2** were docked to the crystal structure of SIRT2 (figure 5.3) (Finnin *et al.* 2001). Compounds **3b**, **5c**, and **5d** were able to adopt binding modes that shared similarities with the best ranked binding conformation of sirtinol. Compound **2** was also able to bind in the same area of the cavity. However, a similar binding pose for **1** could not be found in these dockings.

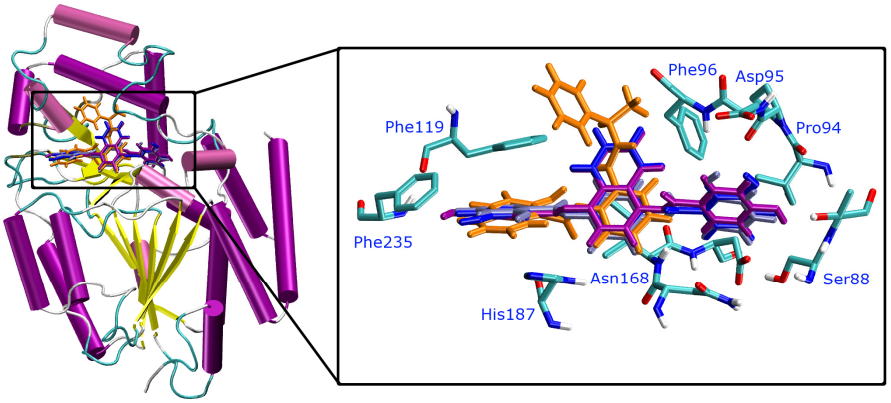


Figure 5.3. Compounds **3b** (iceblue), **5c** (blue), **5d** (purple), and sirtinol (orange) docked into the putative binding site of SIRT2 (Humphrey *et al.* 1996).

5.4 Conclusions

A series of *N*,*N*′-bisbenzylidenebenzene-1,4-diamine and *N*,*N*′-bisbenzylidene-naphthalene-1,4-diamine derivatives were synthesized and tested *in vitro* against SIRT2. The most potent compounds were **3b**, **5c**, and **5d** with IC_{50} of 58.4 μM, 195.9 μM, and 137.4 μM, respectively. Compound **3b** was equipotent with the well-known SIRT2 inhibitor sirtinol. These compounds have a new type of backbone for SIRT2 inhibitors. The new compounds were able to adopt binding modes that shared similarities with the best ranked binding conformation of sirtinol. The synthesized compounds are symmetrical, but there is no requirement of symmetry in the binding site of the SIRT2 inhibitor, and therefore, the next study will involve the synthesis of unsymmetrical compounds.

5.5 Synthetic procedures and analytical data

General method for synthesis of imines 3a–3h and 5a–5g. The reactions were performed in 1–7 mmol scale. The appropriate aldehyde (2.0–2.5 eq) was added to a solution 1,4-phenylenediamine (1.0 eq) in anhydrous ethanol (20 mL) or naphthalene-1,4-diamine (1.0 eq) in anhydrous tetrahydrofuran (except **5c** in anhydrous acetonitrile) (20 mL). The reaction was performed under an argon or nitrogen atmosphere and

protected from light. Benzene-1,4-diamine reactions were stirred at rt or refluxed if necessary. The product precipitated out of the reaction mixture. Naphthalene-1,4-diamine reactions were stirred overnight at rt. Afterwards, the solvent was evaporated and the residue was washed with methanol.

***N,N′*-Bisbenzylidenebenzene-1,4-diamine (3a).** Benzaldehyde (2.1 eq), rt for 1 h (77%). ^{1}H NMR: δ = 7.33 (s, 4 H), 7.48–7.51 (m, 6 H), 7.93–7.95 (m, 4 H), 8.63 (s, 2H). ^{13}C NMR: δ = 121.62, 128.39, 128.42, 131.00, 135.91, 149.16, 159.39. ESI-MS (*m*/*z*): 285 $[M + H]^+$. Anal. ($C_{20}H_{16}N_2$) C, H, N.

***N,N′*-Bis(2-hydroxybenzylidene)benzene-1,4-diamine (3b).** 2-Hydroxybenzaldehyde (2.0 eq), reflux for 3.5 h (99%). The ^{1}H NMR parameters were solved precisely with the PERCHit iterator (Laatikainen *et al.* 1996) using PERCH software version 2004 (Pearch Solutions Ltd, Kuopio, Finland). ^{1}H NMR: δ = 6.977 (d, ^3J = 8.28 Hz, ^4J = 1.06 Hz, 2 H), 6.995 (t, ^3J = 7.70, 7.27 Hz, ^4J = 1.06 Hz, 2 H), 7.426 (td, ^3J = 8.28, 7.27 Hz, ^4J = 1.72 Hz, 2 H), 7.545 (s, 4 H), 7.673 (dd, ^3J = 7.70 Hz, ^4J= 1.72 Hz, 2 H), 9.030 (s, 2 H), 13.068 (s, 2 OH). ^{13}C NMR: δ = 116.51, 119.08, 119.27, 122.44, 132.47, 133.22, 146.58, 160.23, 163.05. ESI-MS (*m*/*z*): 317 $[M + H]^+$. Anal. ($C_{20}H_{16}N_2O_2$) C, H, N.

***N,N′*-Bis(3-Hydroxybenzylidene)benzene-1,4-diamine (3c).** 3-Hydroxybenzaldehyde (2.0 eq), rt for 3 days (80%). ^{1}H NMR: δ = 6.92–6.94 (m, 2 H), 7.30–7.38 (m, 10 H), 8.58 (s, 2 H), 9.66 (s, 2 OH). ^{13}C NMR: δ = 114.18, 118.62, 120.20, 121.88, 129.70, 137.39, 149.18, 157.62, 159.89. ESI-MS (*m*/*z*): 317 $[M + H]^+$. Anal. ($C_{20}H_{16}N_2O_2 \cdot 0.2H_2O$) C, H, N.

***N,N′*-Bis(4-Hydroxybenzylidene)benzene-1,4-diamine (3d).** 4-Hydroxybenzaldehyde (2.0 eq), reflux for 3.0 h (56%). ^{1}H NMR: δ = 6.89 (d, ^3J = 8.5 Hz, 4 H), 7.26 (s, 4 H), 7.78 (d, ^3J = 8.5 Hz, 4 H), 8.51 (s, 2 H), 10.08 (s, 2 OH). ^{13}C NMR: δ = 115.56, 121.66, 127.56, 130.50, 149.22, 158.98, 160.48. ESI-MS (*m*/*z*): 317 $[M + H]^+$. Anal. ($C_{20}H_{16}N_2O_2 \cdot 0.5H_2O$) C, H, N.

***N,N′*-Bis(3,4-Dihydroxybenzylidene)benzene-1,4-diamine (3e).** 3,4-Dihydroxybenzaldehyde (2.1 eq), rt overnight (63%). ^{1}H NMR (DMSO-d_6:CD_3OD): δ = 6.87 (d, ^3J = 8.1 Hz, 2 H), 7.21 (dd, ^3J = 8.1 Hz, ^4J = 1.8 Hz, 2 H), 7.25 (s, 4 H), 7.44–7.45 (m, 2 H), 8.42 (s, 2 H). ^{13}C NMR (DMSO-d_6:CD_3OD): δ = 114.33, 115.63, 121.94, 122.88,

128.50, 145.76, 149.34, 149.56, 159.51. ESI-MS (*m*/*z*): 349 $[M + H]^+$. Anal. ($C_{20}H_{16}N_2O_4 \cdot 0.3H_2O$) C, H, N.

***N,N′*-Bis(2-Methoxybenzylidene)benzene-1,4-diamine (3f).** 2-Methoxybenzaldehyde (2.0 eq), rt for 2 h (95%). ^{1}H NMR: δ = 3.92 (s, 6 H), 7.03–7.06 (m, 2 H), 7.08–7.10 (m, 2 H), 7.26 (s, 4 H), 7.46–7.50 (m, 2 H), 8.06–8.08 (m, 2 H), 8.92 (s, 2 H). ^{13}C NMR: δ = 55.57, 111.53, 120.51, 121.73, 124.12, 126.86, 132.78, 149.93, 154.75, 159.23. ESI-MS (*m*/*z*): 345 $[M + H]^+$. Anal. ($C_{22}H_{20}N_2O_2 \cdot 0.1H_2O$) C, H, N.

***N,N′*-Bis(3-Methoxybenzylidene)benzene-1,4-diamine (3g).** 3-Methoxybenzaldehyde (2.5 eq), reflux 2 h (65%). ^{1}H NMR: δ = 3.84 (s, 6H), 7.10–7.12 (m, 2 H), 7.36 (s, 4 H), 7.43–7.46 (m, 2 H), 7.52–7.53 (m, 4 H) 8.66 (s, 2 H). ^{13}C NMR: δ = 55.14, 112.48, 117.58, 121.54, 121.94, 129.80, 137.47, 149.16, 159.49, 159.82. ESI-MS (*m*/*z*): 345 $[M + H]^+$. Anal. ($C_{22}H_{20}N_2O_2 \cdot 0.1H_2O$) C, H, N.

***N,N′*-Bis(4-Methoxybenzylidene)benzene-1,4-diamine (3h).** 4-Methoxybenzaldehyde (3.7 eq), rt for 3 h (97%). ^{1}H NMR: δ = 3.89 (s, 6 H), 7.00 (d, ^3J = 8.5 Hz, 4 H), 7.25 (s, 4 H), 7.86 (d, ^3J = 8.5 Hz, 4 H), 8.47 (s, 2 H). ^{13}C NMR: δ = 55.69, 114.72, 122.32, 129.97, 130.92, 150.20, 159.32, 162.70. ESI-MS (*m*/*z*): 345 $[M + H]^+$. Anal. ($C_{22}H_{20}N_2O_2 \cdot 0.1H_2O$) C, H, N.

***N,N′*-Bisbenzylidenenaphthalene-1,4-diamine (5a).** Benzaldehyde (2.1 eq), rt overnight (64%). ^{1}H NMR: δ = 7.31 (s, 2 H), 7.57–7.62 (m, 8 H), 8.08–8.10 (m, 4 H), 8.37–8.39 (m, 2 H), 8.76 (s, 2 H). ^{13}C NMR: δ = 113.18, 123.45, 126.21, 128.76, 128.76, 128.92, 131.42, 136.21, 146.18, 159.97. ESI-MS (*m*/*z*): 335 $[M + H]^+$. Anal. ($C_{24}H_{18}N_2 \cdot 0.2H_2O$) C, H, N.

***N,N′*-Bis(2-Hydroxybenzylidene)naphthalene-1,4-diamine (5b).** 2-Hydroxybenzaldehyde (2.1 eq), rt overnight (51%). ^{1}H NMR: δ = 7.03–7.06 (m, 4 H), 7.46–7.50 (s, 2 H), 7.55 (s, 2 H), 7.70–7.72 (m, 2 H), 7.77–7.79 (m, 2 H), 8.25–8.27 (m, 2 H), 9.07 (s, 2 H), 13.06 (s, 2 OH). ^{13}C NMR: δ = 114.73, 116.57, 119.24, 119.73, 122.79, 127.14, 128.23, 132.37, 133.47, 144.16, 160.22, 163.46. ESI-MS (*m*/*z*): 367 $[M + H]^+$. Anal. ($C_{24}H_{18}N_2O_2$) C, H, N.

***N,N′*-Bis(3-Hydroxybenzylidene) naphthalene-1,4-diamine (5c).** 3-Hydroxybenzaldehyde (2.5 eq), rt for 2.5 h (27%).^{1}H NMR: δ = 6.98–6.99 (m, 2 H), 7.26–7.62 (m, 10 H), 8.36 (m, 2 H), 8.68 (s, 2 H), 9.76 (s, 2 OH). ^{13}C NMR: δ =113.32, 114.41, 118.87,

120.46, 123.48, 126.30, 128.96, 129.90, 137.62, 146.16, 157.79, 160.15. ESI-MS (*m*/*z*): 367 $[M + H]^+$. Anal. ($C_{24}H_{18}N_2O_2 \cdot 1.1H_2O$) C, H, N.

***N,N′*-Bis(4-Hydroxybenzylidene)naphthalene-1,4-diamine (5d).** 4-Hydroxybenzaldehyde (2.1 eq), rt for 3 days (47%). ^{1}H NMR: δ = 6.94 (d, ^3J = 8.2 Hz, 4 H), 7.21 (s, 2 H), 7.56–7.58 (m, 2 H), 7.92 (d, ^3J = 8.2 Hz, 4 H), 8.35–8.37 (m, 2 H), 8.60 (s, 2 H), 10.16 (s, 2 OH). ^{13}C NMR: δ = 113.10, 115.74, 123.57, 126.03, 127.87, 129.08, 130.83, 146.23, 159.25, 160.72. ESI-MS (*m*/*z*): 367 $[M + H]^+$. Anal. ($C_{24}H_{18}N_2O_2 \cdot 1.7CH_3OH$) C, H, N. CH_3OH was observed in the NMR spectra.

***N,N′*-Bis(2-Methoxybenzylidene)naphthalene-1,4-diamine (5e).** 2-Methoxybenzaldehyde (2.0 eq), rt overnight (18%). ^{1}H NMR: δ = 3.93 (s, 6 H), 7.12–7.15 (m, 2 H), 7.19–7.21 (m, 4 H), 7.55–7.60 (m, 4 H), 8.23–8.25 (m, 2 H), 8.33–8.35 (m, 2 H), 9.00 (s, 2 H). ^{13}C NMR: δ = 55.74, 111.98, 113.20, 120.70, 123.45, 124.07, 126.19, 127.10, 128.88, 133.16, 146.79, 154.85, 159.25. ESI-MS (*m*/*z*): 395 $[M + H]^+$. Anal. ($C_{26}H_{22}N_2O_2 \cdot 0.1H_2O$) C, H, N.

***N,N′*-Bis(3-Methoxybenzylidene)naphthalene-1,4-diamine (5f).** 3-Methoxybenzaldehyde (2.4 eq), rt for 2 days (60%). ^{1}H NMR ($CDCl_3$): δ = 3.92 (s, 6 H), 7.07–7.09 (m, 4 H), 7.40–7.44 (m, 2 H), 7.52–7.53 (m, 2 H), 7.56–7.58 (m, 2 H), 7.67–7.67 (m, 2 H), 8.38–8.40 (m, 2 H), 8.56 (s, 2 H). ^{13}C NMR ($CDCl_3$): δ = 55.46, 112.17, 112.84, 118.16, 122.40, 123.86, 126.30, 129.27, 129.78, 137.92, 147.27, 159.52, 160.05. ESI-MS (*m*/*z*): 395 $[M + H]^+$. Anal. ($C_{26}H_{22}N_2O_2 \cdot 0.3H_2O$) C, H, N.

***N,N′*-Bis(4-Methoxybenzylidene)naphthalene-1,4-diamine (5g).** 4-Methoxybenzaldehyde (2.0 eq), rt for 2 days (66%). ^{1}H NMR: δ = 3.89 (s, 6 H), 7.07 (d, ^3J = 8.4 Hz, 4 H), 7.19 (s, 2 H), 7.54–7.56 (m, 2 H), 8.00 (d, ^3J = 8.4 Hz, 4 H), 8.36–8.38 (m, 2 H), 8.62 (s, 2 H). ^{13}C NMR: δ = 55.30, 112.86, 114.16, 123.58, 125.90, 129.11, 129.27, 130.50, 146.37, 158.72, 161.99. ESI-MS (*m*/*z*): 395 $[M + H]^+$. Anal. ($C_{26}H_{22}N_2O_2 \cdot 0.1H_2O$) C, H, N.

General method for reduction of imines for amines. The reactions were performed in 1–7 mmol scale. $NaBH_4$ (2.8–3.6 eq) was added carefully in small portions to a solution of **3** (1.0 eq) in 1,2-dimethoxyethane. After the mixture was stirred at rt for 1.5 h to 3 days, a catalytic amount of glacial acetic acid was added according to Krebs and Jørgensen (2002). The mixture was stirred at rt overnight. The solution was acidified to

pH 1–2 using concentrated HCl and stirred for another 10 min. Then the solution was brought to pH 7 using a 2 M NaOH (Lubben and Feringa 1994). The mixture was diluted with water and extracted with CH_2Cl_2. The organic layer was washed once with water, dried over Na_2SO_4 and evaporated. The product was purified by flash chromatography.

***N,N′*-Bisbenzylbenzene-1,4-diamine (4a). 3a** and $NaBH_4$ (2.8 eq), 2 h (43%). ^{1}H NMR: δ = 4.13 (d, ^3J = 5.3 Hz, 4 H), 5.31 (t, ^3J = 5.3 Hz, 2 NH), 6.40 (s, 4 H), 7.17–7.20 (m, 2 H), 7.26–7.29 (m, 4 H), 7.32-7.33 (m, 4 H). ^{13}C NMR: δ = 47.68, 113.71, 126.30, 127.15, 128.01, 140.03, 140.85. ESI-MS (*m*/*z*): 289 $[M + H]^+$. Anal. ($C_{20}H_{20}N_2 \cdot 0.1H_2O$) C, H, N.

***N,N′*-Bis(2-Hydroxybenzyl)benzene-1,4-diamine (4b). 3b** and $NaBH_4$ (2.8 eq), 1.5 h (30%). ^{1}H NMR: δ = 4.08 (s, 4 H), 5.18 (s, 2 NH), 6.44 (s, 4 H), 6.69–6.72 (m, 2 H), 6.76–6.78 (m, 2 H), 6.99–7.03 (m, 2 H), 7.17–7.18 (m, 2 H), 9.47 (s, 2 OH). ^{13}C NMR: δ = 43.10, 114.00, 114.70, 118.59, 126.01, 127.20, 128.28, 140.15, 155.03. ESI-MS (*m*/*z*): 321 $[M + H]^+$. Anal. ($C_{20}H_{20}N_2O_2 \cdot 0.3H_2O$) C, H, N.

***N,N′*-Bis(3-Hydroxybenzyl)benzene-1,4-diamine (4c). 3c** and $NaBH_4$ (2.8 eq), 3 days (36%). ^{1}H NMR: δ = 4.04–4.05 (m, 4 H), 5.26 (s, 2 NH), 6.38 (s, 4 H), 6.57–6.58 (m, 2 H), 6.73–6.74 (m, 4 H), 7.04–7.07 (m, 2 H), 9.18–9.20 (m, 2 OH). ^{13}C NMR: δ = 47.70, 113.32, 113.67, 113.93, 117.73, 128.97, 140.10, 142.46, 157.29. ESI-MS (*m*/*z*): 321 $[M + H]^+$. Anal. ($C_{20}H_{20}N_2O_2 \cdot 0.1H_2O$) C, H, N.

***N,N′*-Bis(4-Hydroxybenzyl)benzene-1,4-diamine (4d). 3d** and $NaBH_4$ (3.6 eq), 3 h (71%). ^{1}H NMR (D_2O): δ = 4.04 (s, 4 H), 6.60 (d, ^3J = 8.4 Hz, 4 H), 6.80 (s, 4H), 7.12 (d, ^3J = 8.4 Hz, 4 H). ^{13}C NMR (D_2O): δ = 51.97, 119.97, 121.52, 127.67, 132.58, 144.12, 168.03. ESI-MS (*m*/*z*): 321 $[M + H]^+$. Anal. ($C_{20}H_{16}N_2O_2 \cdot 2.2H_2O$) C, H, N.

5.6 References

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6 *N*-(3-(4-HYDROXYPHENYL)-PROPENOYL)-AMINO ACID TRYPTAMIDES AS SIRT2 INHIBITORS*

Abstract: A series of *N*-(3-(4-hydroxyphenyl)-propenoyl)-amino acid tryptamides was based on a previously reported new SIRT2 inhibitor from our group, and it was designed to study if the molecular size of the compound could be reduced. The most potent compounds, *N*-(3-(4-hydroxyphenyl)-propenoyl)-2-aminoisobutyric acid tryptamide and *N*-(3-(4-hydroxyphenyl)-propenoyl)-L-alanine tryptamide, were equipotent, 30% smaller in molecular weight, and slightly more selective (SIRT2/SIRT1) than the parent compound.

* Adapted with permission from: Kiviranta PH, Leppänen J, Rinne VM, Suuronen T, Kyrylenko O, Kyrylenko S, Kuusisto E, Tervo AJ, Järvinen T, Salminen A, Poso A, Wallén EAA. *N*-(3-(4-Hydroxyphenyl)-propenoyl)-amino acid tryptamides as SIRT2 inhibitors. Bioorganic & Medicinal Chemistry Letters 17: 2448-51, 2007.

6.1 Introduction

Second virtual screening by Tervo *et al.* (2006) was based on our previous results (Kiviranta *et al.* 2006, Tervo *et al.* 2004) and examinations of favorable interactions between the known SIRT2 inhibitors and the putative binding site. Compound **1** (Tripos 360702) was the most potent compound that was found in the study and it had an IC_{50} value of 51 μM (figure 6.1) (Tervo et al. 2006). Compound **1** had a structural scaffold that was new for SIRT2 inhibitors. The desire to reduce the molecular size of compound **1** was the main focus for this study. The design of the new series of compounds was based on simplifying the structure and finding the essential parts of **1** for the inhibitory activity.

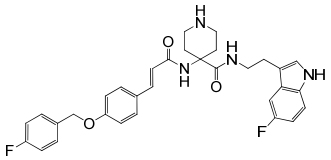
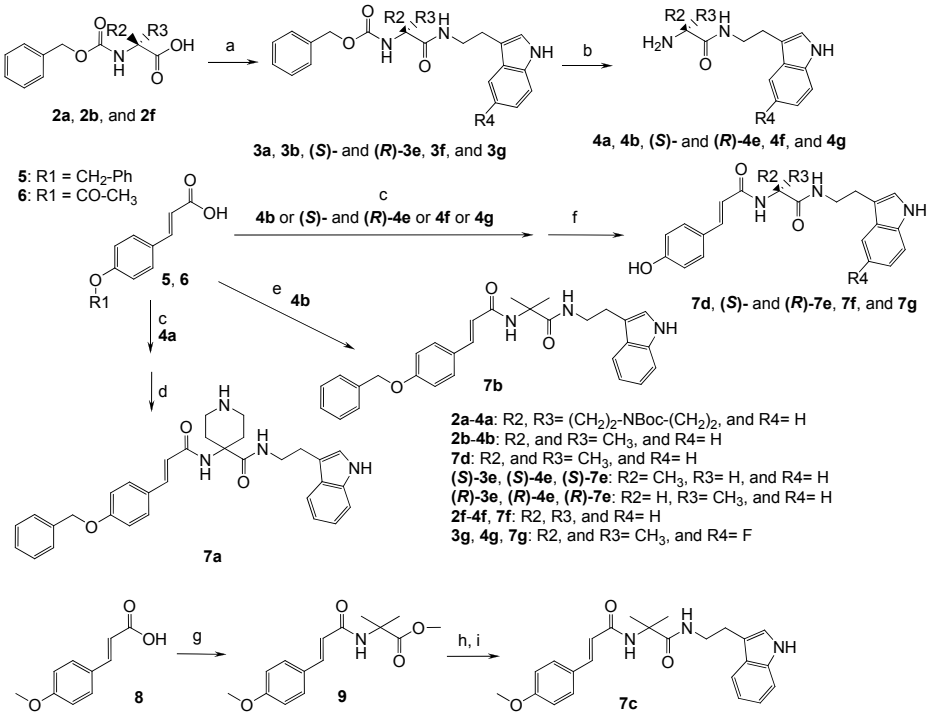


Figure 6.1. Compound **1** found by modelling and virtual screening (Tervo *et al.* 2006).

6.2 Synthetic chemistry

The synthetic routes are presented in scheme 6.1. The starting material 4-amino-1-Boc-piperidine-4-carboxylic acid was synthesized as described in the literature (Wysong *et al.* 1996). The amino groups of the amino acids **2a**, **2b**, and **2f** were protected with benzyl chloroformate. The carboxylic acid was activated with ethyl chloroformate (Sam *et al.* 1959) or DCC (Jung and Gervay 1991) and reacted with tryptamine. *N*-Cbz-L-alanine *N*-hydroxysuccinimide ester and *N*-Cbz-D-alanine *N*-hydroxysuccinimide ester was reacted with tryptamine in THF (Garcia-Lopez *et al.* 1987). The Cbz group was removed with palladium (10%) on activated charcoal and either ammoniumformate or hydrogen gas in methanol. 3-(4-Benzyloxyphenyl)-propenoic acid (**5**) was synthesized from 3-(4-hydroxyphenyl)-propenoic acid and benzyl bromide. 3-(4-Acetoxyphenyl)-propenoic acid (**6**) was synthesized by protecting the phenolic hydroxyl group with acetic anhydride. Compound **7a** was synthesized from **5** with ethyl chloroformate activation followed by reaction with **4a** (Sam *et al.* 1959). The removal of the Boc group yielded compound **7a**. To form compound **7b**, compound **5** was reacted with

oxalyl chloride to form the acid chloride which was reacted with 2-amino-isobutyric acid tryptamide (**4b**) in DCM. Compounds **7d**, (*S*)-**7e**, (*R*)-**7e**, and **7g** were also synthesized from **6** with ethyl chloroformate activation followed by reaction with the appropriate amino acid tryptamide (Sam *et al.* 1959). Compound **7f** was synthesized from compound **6** with DCC activation followed by reaction with glycine tryptamide (**4f**). The acetyl protection group was hydrolyzed with K_2CO_3 in water and methanol or CH_3ONa in methanol, yielding compounds **7d**–**7g**. In most amide formation reactions ethyl chloroformate activation was found to be the most successful method. The yields of the amide bond formations varied between 14–93%.



Scheme 6.1. Reagents a) tryptamine, THF or 1—Et_3N, ethyl chloroformate, DCM or THF, -20–0°C, 2—Et_3N, tryptamine or DMAP, tryptamine, DCC, $CHCl_3$; (b) ammoniumformate, 10% Pd/C, MeOH or H_2, 10% Pd/C, MeOH; (c) 1—Et_3N, ethyl chloroformate, THF or DCM, -20–0°C, 2—Et_3N, **4a**, or **4b**, or (*S*)**-,** or (*R*)-**4e**, or **4g**; or DMAP, **4f**, DCC, DCM and 1,4-dioxane, 0°C; (d) TFA, anisole, sodium thiophenolate; (e) 1—**5**, oxalyl chloride, DMF, DCM; 2—**4b**, Et_3N, DCM; (f) K_2CO_3, H_2O/ MeOH, 0°C or 1M CH_3ONa in MeOH, DCM, 0°C; (g) 1—Et_3N, pivaloylchloride, DCM, 0–25°C, 2—Et_3N, 2-amino-2-methyl-propionic acid methyl ester; (h) $LiOH{\cdot}H_2O$, H_2O/MeOH; (i) 1—Et_3N, ethyl chloroformate, DCM, -20°C, 2—Et_3N, tryptamine.

Another synthetic procedure was used for compound **7c**. 3-(4-Methoxyphenyl)-propenoic acid (**8**) was synthesized as described in the literature (Furniss *et al.* 1989). Compound **8** was activated with pivaloyl chloride and reacted with 2-amino-isobutyric acid methyl ester. The methyl ester of the obtained product **9** was hydrolyzed with $LiOH \cdot H_2O$ in water and methanol. The obtained free carboxylic acid was activated with ethyl chloroformate and reacted with tryptamine to yield compound **7c**. This synthetic procedure was not useful when 3-(4-hydroxyphenyl)-propenoic acid was used because the removal of the methyl ester would also have removed the acetyl protection group of the phenolic hydroxyl group. EX-527 was used as a reference compound. EX-527 was synthesized as described in the literature (Napper *et al.* 2005).

6.3 Results and discussion

The compounds and their inhibitory activities are presented in table 6.1. The inhibitory activities were tested in a Fluor de Lys fluorescence based assay described in Chapter 4.4. Poor water solubility of some compounds was observed when determining the inhibition at higher concentrations. This was the main reason not to determine the IC_{50} values of the compounds with less than 50% inhibition at 200 μM. The importance of the fluorine atoms for the inhibitory activity of **1** was investigated with **7a**. Removal of the fluorine atoms did not have a significant effect on the inhibitory activity. Opening the piperidine ring and removing the amine functionality in the side chain by using a 2-aminoisobutyric acid group in the middle of the structure resulted in **7b**, with a significantly lower inhibitory activity, inhibition of 32.6% at 200 μM. Furthermore, removal of the phenyl group resulted in **7c**, which was slightly more potent, with an inhibition of 55.4% at 200 μM and an IC_{50} of 99 μM. The structure was further simplified by removing the methyl group from the methoxy group, resulting in **7d**. Compound **7d** was equipotent with **1** and it showed that the chemical structure could be reduced in size without affecting the inhibitory activity for SIRT2. The molecular weight of **1** was reduced about 30%. Replacing the aminoisobutyric acid group by an L-alanine group gave (*S*)-**7e**, which had an inhibition of 88.6% at 200 μM and an IC_{50} of 47 μM. Compound (*S*)-**7e** showed also selectivity for SIRT2. However, replacing the aminoisobutyric acid group by a D-alanine group gave (*R*)-**7e**, with an inhibition of

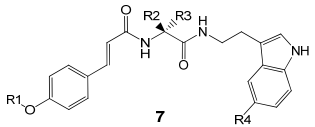
8.5% at 200 μM. The structure was further simplified by replacing the alanine group by a glycine group resulting in **7f**, which was less potent than a L-alanine group but more potent than a D-alanine group. Finally, the effect of the fluorine atom on the indole ring was studied with **7g**. Compound **7g** showed that the fluorine atom does not have a positive effect on the inhibitory activity since **7g** had a slightly lower inhibitory activity compared to **7d**.

In addition, the selectivity for SIRT2 was studied (table 6.1). As earlier reported, SIRT2 inhibitors are also good SIRT1 inhibitors. Interestingly, compound **7d** showed a slightly higher selectivity for SIRT2 than **1**. At the concentration of 200 μM compound **1** inhibits SIRT1 94.1% but **7d** only 6.6%. Compound (*S*)-**7e** is almost as selective as **7d**.

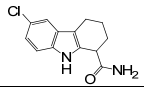
6.4 Conclusions

In conclusion, the essential parts of compound **1** for the inhibitory activity were identified, and the study showed that the molecular weight of compound **1** could be reduced 30% while maintaining the inhibitory activity. In addition, the most potent compounds **7d** and (*S*)-**7e** were slightly more selective for SIRT2 (SIRT2/SIRT1) than compound **1**.

Table 6.1. Compounds and their inhibitory activities for SIRT2 and SIRT1 (95% confidence intervals for IC_{50} given in parentheses).



7

Compd	R1	R2	R3	R4	Inhibition at 200 µM ± SD,[a] % SIRT2	Inhibition at 200 µM ± SD,[a] % SIRT1	IC_{50} (μmol/L) SIRT2[b]	IC_{50} (μmol/L) SIRT1[b]
1	CH_2-Ph-F	$(CH_2)_2$-NH-$(CH_2)_2$		F	77.3 ± 4.8	94.1 ± 3.5	51 (27–75)	73 (47-114)
7a	CH_2-Ph	$(CH_2)_2$-NH-$(CH_2)_2$		H	79.1 ± 1.4	97.0 ± 1.9	63 (41–96)	52 (38–70)
7b	CH_2-Ph	CH_3	CH_3	H	32.6 ± 18.2	10.2 ± 6.4	-	-
7c	CH_3	CH_3	CH_3	H	55.4 ± 3.3	10.3 ± 4.3	99 (66–150)	-
7d	H	CH_3	CH_3	H	83.3 ± 3.7	6.6 ± 1.8	50 (23-109)	-
(*S*)-**7e**	H	CH_3	H	H	88.6 ± 0.8	17.7 ± 1.0	47 (28–79)	-
(*R*)-**7e**	H	H	CH_3	H	8.5 ± 6.0	3.2 ± 5.5	-	-
7f	H	H	H	H	44.3 ± 10.6	9.9 ± 1.2	-	-
7g	H	CH_3	CH_3	F	67.5 ± 1.1	7.0 ± 4.0	80 (53–120)	-
10 EX-527[c]					89.4 ± 2.8	98.9 ± 0.3	14 (8-25)	0.28 (0.23-0.34)

[a] SD = standard deviation, [b] IC_{50} were determined for compounds which had over 50% inhibition at 200 µM for SIRT2 or SIRT1. [c] (Napper *et al.* 2005)

6.5 Synthetic procedures and analytical data

Synthesis of 7a

4-Amino-1-Boc-piperidine-4-carboxylic acid. The reaction had three steps and they were done according to Wysong *et al.* (1996).

Step 1: 4-Piperidone monohydrate hydrochloride 53.55 g (348.6 mmol) and $(NH_4)_2CO_3$ 69.0 g (718.5 mmol) were dissolved in the mixture of H_2O (180 mL) and MeOH (220 mL). NaCN 34.0 g (693.7 mmol) was dissolved in H_2O (100 mL) and added to the well stirred mixture during 5 min. A light precipitate started to form immediately. The mixture was protected with a light plastic cap and stirred at rt for 3 d. The precipitated product was filtered and washed with H_2O. After the vacuum, the product still contained some $(NH_4)_2CO_3$. It was stirred in H_2O for a couple of hours, filtered and dried well in the vacuum (75%).

Step 2: Piperidine-4-spriro-5'-hydantoin 41.3 g (244.1 mmol) was dissolved in 1,2-dimethoxyethane. The suspension was formed. Di-tert-butyl dicarbonate 275.3 g (1.26 mol), DMAP 0.53 g (4.32 mmol) and Et_3N 34.6 mL (247.9 mmol) were added. The mixture was stirred at rt for 3.5 h. Carbon dioxide was freed. The second portion of DMAP 0.53 g (4.32 mmol) was added and the mixture was stirred at rt for 6 d. The third portion of DMAP 0.53 g (4.32 mmol) was added and the mixture was stirred at rt overnight. The solvent was evaporated and the residue was dissolved in 800 mL of DCM. The organic phase was washed twice with 1 M HCl, once with sat. Na_2CO_3 aq. and once with sat. NaCl aq. The organic phase was dried, filtered and evaporated. The residue was yet first stirred in 200 mL of EtOH and evaporated until dryness. Then, it was stirred in the mixture of EtOH (250 mL) and H_2O (750 mL). The crystals were filtered and washed with 200 mL of the 25% EtOH:H_2O mixture and, finally, with 500 mL of H_2O. The product was dried in the vacuum for several days (86%). ^{1}H NMR ($CDCl_3$): δ = 1.47 (s, 9 H), 1.55 (s, 9 H), 1.59 (s, 9 H), 1.73–1.76 (m, 2 H), 2.64–2.70 (m, 2 H), 3.37–3.47 (m, 2 H), 4.04–4.19 (m, 2 H).

Step 3: 1-Boc-piperidine-4-spiro-5'-(1',3'-*bis*-Boc)hydantoin 34.0 g (72.4 mmol) was dissolved in 450 mL of 1,2-dimethoxyethane. The suspension was formed. NaOH 26.0 g (650 mmol) was dissolved in 650 mL of H_2O and added. The mixture was stirred at rt for 2 d. Diethyl ether 200 mL was added and the mixture was stirred for 0.5 h. The

phases were separated and the procedure was repeated once. The H_2O phase was neutralized by adding first sat. HCl aq. and, then 2 M HCl until the pH was about 7–8. The product was crystallized during 4 d at 4 ^oC. The crystals were filtered, washed with small amount of H_2O and dried in the vacuum (76%).

4-(Cbz-amino)-1-Boc-piperidine-4-carboxylic acid (2a). 4-Amino-1-Boc-piperidine-4-carboxylic acid 8.3 g (34.0 mmol) was dissolved in the mixture of 1,4-dioxane:water (1:2) where 5% K_2CO_3 (0.36 M) was added. Benzyl chloroformate 9.7 mL (68.0 mmol) was dissolved in a small amount of 1,4-dioxane and added to the mixture. The mixture was stirred at rt overnight. Afterward, diethyl ether and sat. $NaHCO_3$ aq. were added. Phases were separated and the organic phase was yet extracted once with sat. $NaHCO_3$ aq. The water phases were combined and made acidic with 2 M HCl. The product was extracted with DCM, dried and evaporated (78%). ^1H NMR (DMSO-d_6): δ = 1.39 (s, 9 H), 1.72–1.78 (m, 2 H), 1.93–1.96 (m, 2 H), 3.02 (s, 2 H), 3.65–3.68 (m, 2 H), 5.02 (s, 2 H), 7.30–7.36 (m, 5 H), 12.51 (s, OH).

4-(Cbz-amino)-1-Boc-piperidine-4-carboxylic acid tryptamide (3a). Compound **2a** 3.78 g (10.0 mmol) and Et_3N 1.53 mL (11.0 mmol) were dissolved in dry DCM and ethyl chloroformate 0.95 mL (10.0 mmol) was added to active the carboxylic acid during 1 h at -20^{o}C under argon. Another portion of Et_3N 1.53 mL (11.0 mmol) and tryptamine 1.76 g (11.0 mmol) were added and then the mixture was stirred under argon at rt overnight (Sam *et al.* 1959). The mixture was washed twice with 30% citric acid, once with sat. NaCl aq. and once with sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified by chromatography on silica gel using manual gradient of ethyl acetate:petrol ether (50:50→66:33→100:0) (67%). ^1H NMR ($CDCl_3$): δ = 1.44 (s, 9 H), 1.90 (s, 2 H), 2.00–2.04 (m, 2 H), 2.92 (s, 2 H), 2.98–3.03 (m, 2 H), 3.56–3.57 (m, 2 H), 3.77–3.80 (m, 2 H), 5.01 (s, 2 H), 6.95 (s, 1 H), 7.09–7.12 (m, 1 H), 7.17–7.20 (m, 1 H), 7.30–7.37 (m, 6 H), 7.57–7.59 (m, 1 H).

4-Amino-1-Boc-piperidine-4-carbocylic acid tryptamide (4a). Compound **3a** 6.63 g (12.7 mmol) was dissolved in dry MeOH and ammoniumformate 2.45 g (38.8 mmol) and 10% Pd/C 0.47 g (4.37 mmol) were added. The mixture was stirred under argon at the rt for 3 h and, then, it was filtered through Celite and the solvent was evaporated. The residue was dissolved in DCM and washed with sat. Na_2CO_3 aq. The organic phase

was dried and evaporated (89%). ^{1}H NMR (CD_3OD): δ = 1.28–1.31 (m, 2 H), 1.45 (s, 9 H), 1.90–1.96 (m, 2 H), 2.96 (t, ^3J = 7.1 Hz, 2 H), 3.15 (s, 2 H), 3.50 (t, ^3J = 7.1 Hz, 2 H), 3.73–3.77 (m, 2 H), 6.98–7.01 (m, 1 H), 7.07–7.09 (m, 2 H), 7.32–7.33 (m, 1 H), 7.56–7.57 (m, 1 H).

3-(4-Benzyloxyphenyl)-propenoic acid (5). 3-(4-Hydroxyphenyl)-propenoic acid 4.0 g (24.4 mmol) was dissolved in EtOH 120 mL and KOH 6.83 g (121.8 mmol) was dissolved in water 80 mL. These mixtures were combined. Benzyl bromide 5.79 mL (48.7 mmol) was dissolved in EtOH 40.0 mL and added slowly to the mixture through the dropping funnel. The mixture was stirred at rt for 3 d. The mixture was poured into ice-water and acidified with 3 M HCl. The product precipitated out the solution. The product was filtered, washed with water and recrystallized from hot toluene (41%). ^{1}H NMR (DMSO-d_6): δ = 5.15 (s, 2 H), 6.37 (d, ^3J = 16.0 Hz, 1 H), 7.04 (d, ^3J = 8.8 Hz, 2 H), 7.32–7.35 (m, 1 H), 7.38–7.41 (m, 2 H), 7.44–7.46 (m, 2 H), 7.54 (d, ^3J = 16.0 Hz, 1 H), 7.62 (d, ^3J = 8.7 Hz, 2 H), 12.19 (s, OH).

4-(3-(4-Benzyloxyphenyl)-propenoyl-amino)-1-Boc-piperidine-4-carboxylic acid tryptamide. Compound **5** 0.50 g (1.96 mmol) and Et_3N 0.30 mL (2.16 mmol) were dissolved in dry THF. Ethyl chloroformate 0.21 mL (2.16 mmol) was added to active the carboxylic acid during 1 h at -20°C under argon. Another portion of Et_3N 0.30 mL (2.16 mmol) and **4a** 0.76 g (1.96 mmol) were added and then the mixture was stirred under argon at the rt for 3 d (Sam *et al.* 1959). The solvents were evaporated and the residue was dissolved in DCM and washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was recrystallized from MeOH (41%). ^{1}H NMR (CD_3OD): δ = 1.46 (s, 9 H), 1.94–1.98 (m, 2 H), 2.04–2.07 (m, 2 H), 2.94 (t, ^3J = 7.1 Hz, 2 H), 3.07–3.12 (m, 2 H), 3.50 (t, ^3J = 7.2 Hz, 2 H), 3.79–3.82 (m, 2 H), 6.55–6.58 (m, 1 H), 6.96–6.99 (m, 1 H), 7.02–7.07 (m, 4 H), 7.26–7.28 (m, 1 H), 7.31–7.33 (m, 1 H), 7.36–7.39 (m, 2 H), 7.43–7.46 (m, 3 H), 7.50–7.52 (m, 2 H), 7.55–7.57 (m, 1 H).

4-(3-(4-Benzyloxyphenyl)-propenoyl-amino)-piperidine-4-carboxylic acid tryptamide (7a). 4-(3-(4-Benzyloxyphenyl)-propenoyl-amino)-1-Boc-piperidine-4-carboxylic acid tryptamide 0.45 g (0.72 mmol) was dissolved in DCM (10 mL). Methoxybenzol 0.20 mL (1.84 mmol) and sodium thiophenolate 0.02 g (0.11 mmol)

were added to the solution (Guerlavais *et al.* 2003). Trifluoroacetic acid 2 ml (0.03 mmol) was added at -20°C. Because the reaction did not proceed, the -20°C bath was changed to the 0°C bath. The reaction was completed in 1 h. The solvents were evaporated and the residue was dissolved in diethyl ether. The product precipitated out of the solution. The product was yet dissolved in ethyl acetate and washed twice with $NaHCO_3$ (5%), dried and evaporated. The product was purified by Chromatotron® on silica gel using MeOH:NH_4OH (98:2) and recrystallized from acetone (8%). ^{1}H NMR (CD_3OD): δ = 1.96–2.01 (m, 2 H), 2.05–2.07 (m, 2 H), 2.75–2.81 (m, 2 H), 2.86–2.90 (m, 2 H), 2.94 (t, ^3J = 7.2 Hz, 2 H), 3.51 (t, ^3J = 7.2 Hz, 2 H), 5.12 (s, 2 H), 6.56–6.59 (m, 1 H), 6.96–7.06 (m, 5 H), 7.26–7.32 (m, 2 H), 7.36–7.38 (m, 2 H), 7.42–7.45 (m, 3 H), 7.50–7.51 (m, 2 H), 7.55–7.57 (m, 1 H). ^{13}C NMR (CD_3OD): δ = 26.12, 33.40, 41.44, 42.37, 59.72, 71.10, 112.22, 113.51, 116.35, 119.32, 119.41, 119.59, 122.29, 123.62, 128.59, 128.74, 129.00, 129.16, 129.56, 130.57, 138.16, 138.37, 141.91, 161.75, 168.64, 176.25. ESI-MS (m/z): 523 $[M + H]^+$. Anal. ($C_{32}H_{34}N_4O_3 \cdot 0.1H_2O$) C, H, N.

Synthesis of 7b

2-(Cbz-amino)-isobutyric acid (2b) was prepared from 2-amino-2-methyl-propionic acid 3.0 g (29 mmol) and benzyl chloroformate 8.3 mL (58 mmol) according to the syntheses of **2a**. The product was used directly for the following coupling reaction. ^{1}H NMR ($(CD_3)_2CO$): δ = 1.52 (s, 6 H), 5.04 (s, 2 H), 6.54 (s, NH), 7.27–7.37 (m, 5 H), 10.91 (s, OH).

2-(Cbz-amino)-isobutyric acid tryptamide (3b) was prepared from acid **2b** 1.5 g (6.32 mmol) and tryptamine 1.11 g (6.95 mmol) using ethyl chloroformate 0.60 mL (6.32 mmol) and two portions of Et_3N 1.9 mL (13 mmol) in dry DCM according to the syntheses of **3a** (Sam *et al.* 1959). The mixture was washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. Some of the product was already crystallized during citric acid wash. The organic phase was dried and evaporated. The product was recrystallized from ethyl acetate and hexane (49%). ^{1}H NMR ($CDCl_3$): δ = 1.46 (s, 6 H), 2.93–2.96 (m, 2 H), 3.56–3.60 (m, 2 H), 5.04 (s, 2 H), 6.97 (s, 1 H), 7.10–7.13 (m, 1 H), 7.18–7.21 (m, 1 H), 7.32–7.37 (m, 5 H), 7.59–7.61 (m, 1 H), 7.88 (s, 1 H).

2-Amino-isobutyric acid tryptamide (4b) was prepared from compound **3b** 1.2 g (3.1 mmol) using ammoniumformate 0.58 g (9.3 mmol) and 10% Pd/C 0.10 g (1.0 mmol) according to the syntheses of **4a**. The mixture was stirred overnight (98%). ^{1}H NMR (CD_3OD): δ = 1.25 (s, 6 H), 2.96 (t, ^3J = 7.2 Hz, 2 H), 3.49 (t, ^3J = 7.2 Hz, 2 H), 6.98–7.01 (m, 1 H), 7.06–7.10 (m, 2 H), 7.32–7.33 (m, 1 H), 7.57–7.58 (m, 1 H).

3-(4-Benzyloxyphenyl)-propenoic acid chloride. Compound **5** 0.31 g (1.22 mmol) was dissolved in dry DCM and a couple of drops DMF was added as a catalyser. Oxalyl chloride 0.47 mL (5.38 mmol) was added carefully to the mixture. The mixture was stirred at rt for 2.5 h, the solvent was evaporated and the product was dried well in the vacuum. The product was used immediately without further purification.

2-(3-(4-Benzyloxyphenyl)-propenoyl-amino)-isobutyric acid tryptamide (7b). Compound **4b** 0.3 g (1.22 mmol) was dissolved in dry DCM and Et_3N 0.19 mL (1.35 mmol) was added. 3-(4-Benzyloxyphenyl)-propenoic acid chloride was dissolved in dry DCM and added to the mixture. The mixture was stirred under argon at rt overnight. The mixture was washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The solvent was dried and evaporated. The product was purified by the chromatography on silica gel twice using ethyl acetate and recrystallized from ethyl acetate (14%). ^{1}H NMR (CD_3OD): δ = 1.48 (s, 6 H), 2.95 (t, ^3J = 7.2 Hz, 2 H), 3.50 (t, ^3J = 7.3 Hz, 2 H), 5.12 (s, 2 H), 6.44–6.48 (m, 1 H), 6.98–7.07 (m, 5 H), 7.27–7.32 (m, 2 H), 7.36–7.44 (m, 5 H), 7.48–7.50 (m, 2 H), 7.57–7.59 (m, 1 H). ^{13}C NMR (CD_3OD): δ = 25.67, 25.89, 41.38, 57.79, 70.92, 112.08, 112.99, 116.11, 119.16, 119.41, 119.45, 122.15, 123.42, 128.36, 128.47, 128.82, 128.95, 129.38, 130.31, 137.85, 138.01, 141.41, 161.38, 168.07, 176.73. ESI-MS (m/z): 482 $[M + H]^+$. Anal. ($C_{30}H_{31}N_3O_3 \cdot 0.1H_2O$) C, H, N.

Synthesis of 7c

2-Amino-isobutyric acid methyl ester. 2-Amino-2-methyl-propionic acid 3.0 g (29 mmol) was dissolved in MeOH. Thionylchloride 5.3 mL (73 mmol) was dissolved in MeOH and added to the mixture through the dropping funnel at 0°C. The mixture was refluxed for 3 h, evaporated and dried in the vacuum. The product was used without further purification. ^{1}H NMR (DMSO-d_6): δ = 1.51 (s, 6 H), 3.76 (s, 3 H), 8.80 (s, NH2).

3-(4-Methoxyphenyl)-propenoic acid (8). 4-Methoxy-benzaldehyde 0.89 mL (7.3 mmol), malonic acid 1.7 g (16 mmol), pyridine 3.3 mL (41 mmol) and a couple of drops piperidine were refluxed for 1 h. After cooling the reaction flask, the mixture was poured into excess water containing enough HCl (>1.3 mL (41 mmol)) to combine with the pyridine, filtered and washed with H_2O. The product was recrystallized three times from EtOH (31%) (Furniss *et al.* 1989). ^{1}H NMR (DMSO-d_6): δ = 3.78 (s, 3 H), 6.37 (d, ^3J = 16.0 Hz, 1 H), 6.96 (d, ^3J = 8.7 Hz, 2 H), 7.55 (d, ^3J = 16.0 Hz, 1 H), 7.62 (d, ^3J = 8.7 Hz, 2 H), 12.2 (s, OH).

2-(3-(4-Methoxyphenyl)-propenoyl-amino)-isobutyric acid methyl ester (9). Acid **8** 2.5 g (14 mmol) and Et_3N 2.1 mL (15 mmol) were dissolved in DCM. Pivaloylchloride 1.7 mL (14 mmol) was dissolved in DCM and added to the mixture through the dropping funnel at 0°C. The mixture was stirred for 1 h. Another portion of Et_3N 2.1 mL (15 mmol) was added. 2-Amino-isobutyric acid methyl ester 1.6 g (14 mmol) was dissolved in DCM and added to the mixture at rt. The mixture was stirred for 4 h. The mixture was washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The solvent was dried and evaporated. The product was purified by chromatography on silica column using manual gradient of DCM:MeOH (99:1→98:2) (43%). ^{1}H NMR ($CDCl_3$): δ = 1.63 (s, 6 H), 3.78 (s, 3 H), 3.83 (s, 3 H), 6.17 (s, NH), 6.27 (d, ^3J = 15.6 Hz, 1 H), 6.88 (d, ^3J = 8.8 Hz, 2 H), 7.44 (d, ^3J = 8.7 Hz, 2 H), 7.55 (d, ^3J = 15.6 Hz, 1 H).

2-(3-(4-Methoxyphenyl)-propenoyl-amino)-isobutyric acid. Ester **9** 1.7 g (6.0 mmol) was dissolved in the mixture of MeOH 72 mL and $LiOH·H_2O$ 1.5 g (36 mmol) in H_2O 24 mL. The mixture was stirred at rt for 5 h. MeOH was evaporated and more H_2O was added. The mixture was washed twice with diethyl ether and, then the water phase was made acidic with 3 M HCl. The product started to precipitate when it was extracted from H_2O to diethyl ether. The diethyl ether phase was evaporated (87%). ^{1}H NMR ($CDCl_3$): δ = 1.64 (s, 6 H), 3.82 (s, 3 H), 6.37 (d, ^3J = 15.5 Hz, 1 H), 6.87 (d, ^3J = 8.7 Hz, 2 H), 7.45 (d, ^3J = 8.7 Hz, 2 H), 7.61 (d, ^3J = 15.6 Hz, 1 H).

2-(3-(4-Methoxyphenyl)-propenoyl-amino)-isobutyric acid tryptamide (7c) was prepared from 2-(3-(4-methoxyphenyl)-propenoyl-amino)-isobutyric acid 1.4 g (5.2 mmol) and tryptamine 0.84 g (5.2 mmol) using ethyl chloroformate 0.50 mL (5.2 mmol)

and two portions of Et_3N 1.6 mL (12 mmol) in dry DCM according to the syntheses of **4a**. The mixture was stirred at rt for 2 d (Sam *et al.* 1959). EtOH was added to clarify the mixture before it was washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was evaporated and the product was purified by chromatography on silica column using manual gradient of DCM:MeOH (99:1→98:2→97:3) (27%). ^{1}H NMR ($(CD_3)_2CO$): δ = 1.53 (s, 6 H), 2.93 (t, ^3J = 7.2 Hz, 2 H), 3.50 (q, ^3J = 6.8 Hz, 2 H), 3.83 (s, 3 H), 6.56–6.59 (m, 1 H), 6.95–7.01 (m, 3 H), 7.05–7.08 (m, 1 H), 7.17 (s, 1 H), 7.34–7.37 (m, 3 H), 7.44–7.47 (m, 1 H), 7.51–7.53 (m, 2 H), 7.60–7.61 (m, 1 H), 9.96 (s, NH). ^{13}C NMR ($(CD_3)_2CO$): δ = 25.74, 26.23, 41.01, 55.70, 57.74, 112.07, 112.12, 113.48, 115.14, 119.35, 119.37, 120.91, 122.04, 123.40, 123.56, 128.84, 130.06, 139.96, 161.18, 165.97, 174.99. ESI-MS (m/z): 406 [M + H]$^+$. Anal. ($C_{24}H_{27}N_3O_3 \cdot 0.1H_2O$) C, H, N.

Synthesis of 7d

3-(4-Acetoxyphenyl)-propenoic acid (6). 3-(4-Hydroxyphenyl)-propenoic acid 13 g (80 mmol) was dissolved in pyridine and acetic anhydride 19 mL (200 mmol) was added. The mixture was stirred at rt overnight. The mixture was poured into the ice-water (100 mL) and made acidic with 3 M HCl. The precipitated product was filtered and washed with water and diethyl ether (86%). ^{1}H NMR (CD_3OD): δ = 2.28 (s, 3 H), 6.44–6.48 (m, 1 H), 7.16–7.18 (m, 2 H), 7.65–7.68 (m, 3 H).

2-(3-(4-Acetoxyphenyl)-propenoyl-amino)-isobutyric acid tryptamide was prepared from compound **6** 0.77 g (3.7 mmol) and compound **4b** 0.91 g (3.7 mmol) using ethyl chloroformate 0.35 mL (3.7 mmol) and two portions of Et_3N 1.1 mL (8.2 mmol) in dry THF according to the syntheses of **3a** (Sam *et al.* 1959). The solvent was evaporated. The residues was dissolved in DCM and washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified twice by chromatography on silica column using manual gradients of ethyl acetate:MeOH (95:5→90:10→0:100) and DCM:MeOH (97:3→95:5) (93%). ^{1}H NMR (CD_3OD): δ = 1.49 (s, 6 H), 2.30 (s, 3 H), 2.95–2.98 (m, 2 H), 3.50–3.53 (m, 2 H), 6.53–6.56 (m, 1 H), 6.98–7.01 (m, 1 H), 7.05–7.08 (m, 2 H), 7.12–7.14 (m, 2 H), 7.28–7.30 (m, 1 H), 7.44–7.48 (m, 1 H), 7.56–7.59 (m, 3 H).

2-(3-(4-Hydroxyphenyl)-propenoyl-amino)-isobutyric acid tryptamide (7d). 2-(3-(4-Acetoxyphenyl)-propenoyl-amino)-isobutyric acid tryptamide 0.80 g (1.9 mmol) was dissolved in the mixture of H_2O (20 mL) and MeOH (20 mL) at 0°C. K_2CO_3 0.28 g (20 mmol) was added. Compound was dissolved fully when more MeOH (20 mL) was added. The mixture was stirred 10 min on an ice bath and, then overnight at rt. Next day, MeOH was evaporated and the residue was dissolved in DCM and a small amount of concentrated NaCl and stirred for a while. The phases were separated and the water phase was yet extracted once with the mixture of $CHCl_3$ and EtOH (1:1). The product was purified twice by chromatography on silica column using first only ethyl acetate and then manual gradient of ethyl acetate and petrol ether (50:50→80:20→100:0) (41%). ^{1}H NMR ($(CD_3)_2CO$): δ = 1.55 (s, 6 H), 2.93–2.96 (m, 2 H), 3.50–3.54 (m, 2 H), 6.49–6.52 (m, 1 H), 6.85–6.87 (m, 2 H), 6.97–7.00 (m, 1 H), 7.04–7.08 (m, 1 H), 7.14–7.14 (m, 1 H), 7.32–7.34 (m, 1 H), 7.39–7.47 (m, 5 H), 7.58–7.60 (m, 1 H), 8.88 (s, OH), 9.93 (s, NH). ^{13}C NMR ($(CD_3)_2CO$): δ = 25.79, 26.20, 41.04, 57.72, 112.12, 113.41, 116.63, 119.33, 119.35, 120.00, 122.02, 123.56, 127.73, 129.58, 130.24, 137.74, 140.41, 159.84, 166.26, 175.18. ESI-MS (m/z): 392 $[M + H]^+$. Anal. ($C_{23}H_{25}N_3O_3 \cdot 0.3H_2O$) C, H, N.

Synthesis of (*S*)-7e

***N*-Cbz-L-Alanine tryptamide ((*S*)-3e).** The reaction was done according to Garcia-López *et al.* (1987). *N*-Cbz-L-Alanine *N*-hydroxysuccinimide ester 1.2 g (3.8 mmol) and tryptamine 0.93 g (5.6 mmol) were reacted in dry THF under argon at rt overnight. The solvent was evaporated and the residue was dissolved in DCM and washed twice with 30% citric acid, once with sat. NaCl aq. and twice sat. $NaHCO_3$ aq. DCM was dried and evaporated. The product was purified by CombiFlash® chromatography on siliga column using DCM:MeOH (99:1) (78%). ^{1}H NMR (DMSO-d_6): δ = 1.19 (d, ^3J = 7.2 Hz, 3 H), 2.82 (t, ^3J = 7.3 Hz. 2 H), 3.28–3.38 (m, 2 H), 3.99–4.05 (m, 1 H), 4.99–5.07 (m, 2 H), 6.96–6.99 (m, 1 H), 7.05–7.08 (m, 1 H), 7.14 (s, 1 H), 7.31–7.38 (m, 6 H), 7.54–7.55 (m, 1 H), 7.92–7.94 (m, 1 NH), 10.80 (s, NH).

L-Alanine tryptamide ((*S*)-4e). Compound (*S*)-**3e** 1.1 g (3.0 mmol) was dissolved in dry MeOH and 10% Pd/C 0.11 g (1.0 mmol) was added. Hydrogen gas was led into the reaction flask from the hydrogen balloon at rt for 1 h. The mixture was filtered through

Celite and the solvent was evaporated (98%). ^{1}H NMR (DMSO-d_6): δ = 1.10 (d, ^3J = 6.9 Hz, 3 H), 2.82 (t, ^3J = 7.4 Hz, 2 H), 3.20–3.24 (m, 1 H), 3.32–3.37 (m, 2 H), 6.96–6.99 (m, 1 H), 7.04–7.08 (m, 1 H), 7.14–7.15 (m, 1 H), 7.32–7.34 (m, 1 H), 7.54–7.56 (m, 1 H), 7.87–7.98 (m, 1 NH), 10.80 (s, NH).

***N*-(3-(4-Acetoxyphenyl)-propenoyl)-L-alanine tryptamide** was prepared from acid **6** 0.56 g (2.7 mmol) and compound (*S*)-**4e** 0.66 g (2.9 mmol) using ethyl chloroformate 0.26 mL (2.7 mmol) and two portions of Et_3N 0.82 mL (6.0 mmol) in dry THF according to the syntheses of **3a** (Sam *et al.* 1959). The carboxylic acid activation was done at 0°C. THF was evaporated and the residue was dissolved in DCM and washed with 30% citric acid, sat. NaCl aq. and sat. $NaHCO_3$ aq. The organic phase was dried and evaporated (47%). ^{1}H NMR (DMSO-d_6): δ = 1.25 (d, ^3J = 7.1 Hz, 3 H), 2.28 (s, 3 H), 2.84 (t, ^3J = 7.4 Hz, 2 H), 3.34–3.38 (m, 2 H), 4.38–4.44 (m, 1 H), 6.74–6.77 (m, 1 H), 6.96–6.99 (m, 1 H), 7.04–7.07 (m, 1 H), 7.15–7.19 (m, 3 H), 7.33–7.34 (m, 1 H), 7.43–7.46 (m, 1 H), 7.54–7.56 (m, 1 H), 7.60–7.62 (m, 2 H), 8.08–8.10 (m, NH), 8.26–8.28 (m, NH), 10.80 (s, NH).

***N*-(3-(4-Hydroxyphenyl)-propenoyl)-L-alanine tryptamide ((*S*)-7e).** *N*-(3-(4-Acetoxyphenyl)-propenoyl)-L-alanine tryptamide 0.53 g (1.3 mmol) was dissolved in DCM. A small amount of MeOH was added to dissolve the substance completely. The reaction flask was under argon at 0°C. 2.5 mL (2.5 mmol) of 1 M CH_3ONa in MeOH was added (Boiadjiev and Lightner 1996). Color of the mixture was immediately changed to yellow and the reaction was over in 5 min. DCM was evaporated and the residue was dissolved in MeOH and acidified with 3 M HCl. H_2O was added dropwise to precipitate the product. The product was filtered and washed with diethyl ether and DCM (32%). ^{1}H NMR (CD_3OD): δ = 1.34 (d, ^3J = 7.1 Hz, 3 H), 2.96 (t, ^3J = 7.2 Hz, 2 H), 3.30–3.32 (m, 2 H), 4.42 (q, ^3J = 7.1 Hz, 1 H), 6.43–6.46 (m, 1 H), 6.79–6.81 (m, 2 H), 6.97–7.00 (m, 1 H), 7.05–7.08 (m, 2 H), 7.29–7.31 (m, 1 H), 7.40–7.48 (m, 3 H), 7.55–7.57 (m, 1 H). ^{13}C NMR (CD_3OD): δ = 18.30, 25.97, 41.18, 50.38, 112.10, 112.86, 116.58, 117.82, 119.10, 119.50, 122.18, 123.41, 127.48, 128.47, 130.52, 137.88, 142.28, 160.32, 168.64, 174.79. ESI-MS (m/z): 378 $[M + H]^+$. Anal. ($C_{22}H_{23}N_3O_3 \cdot 0.3H_2O$) C, H, N.

Synthesis of (*R*)-7e

***N*-Cbz-D-Alanine N-hydroxysuccinimide ester.** (*R*)-2-Benzyloxycarbonyl-amino-propionic acid 2.0 g (9.0 mmol) and *N*-hydroxysuccinimide 1.0 g (9.0 mmol) were dissolved in dry acetonitrile. DCC 1.9 g (9.0 mmol) was dissolved in dry acetonitrile and added to the mixture through the dropping funnel under argon at -20°C. The reaction was reacted overnight at -20°C. The precipitate was filtered off and the solvent was evaporated. The product was recrystallized from hexane and dissolved in THF. Urea did not dissolve in THF and it was easy to remove by filtration. The solvent was evaporated (57%). ^{1}H NMR (DMSO-d_6): δ = 1.45 (d, ^3J = 7.3 Hz, 3 H), 2.82 (s, 4 H), 4.53 (q, ^3J = 7.2 Hz, 1 H), 5.07 (s, 2 H), 7.33–7.40 (m, 4 H), 8.08–8.09 (m, 1H).

***N*-Cbz-D-Alanine tryptamide ((*R*)-3e)** was prepared from compound *N*-Cbz-D-alanine *N*-hydroxysuccinimide ester 1.6 g (5.1 mmol) and tryptamine 1.3 g (7.6 mmol) according to Garcia-López *et al.* (1987) and the syntheses of (*S*)-**3e**. The product was washed four times with 30% citric acid, once with sat. NaCl aq. and three times with sat. $NaHCO_3$ aq. The product was purified by chromatography on siliga column using ethyl acetate (71%). ^{1}H NMR (CD_3OD): δ = 1.26–1.28 (m, 3 H), 2.92–2.94 (m, 2 H), 3.43–3.54 (m, 2 H), 4.07–4.12 (m, 1 H), 5.03–5.10 (m, 2 H), 6.98–7.09 (m, 3 H), 7.28–7.34 (m, 6 H), 7.55–7.56 (m, 1 H).

D-Alanine tryptamide ((*R*)-4e) was prepared from (*R*)-**3e** 1.3 g (3.6 mmol) and 10% Pd/C 0.13 g (1.2 mmol) according to the syntheses of (*S*)-**4e** for 0.5 h. The product was used without further purification. ^{1}H NMR (CD_3OD): δ = 1.20 (d, ^3J = 6.9 Hz, 3 H), 2.94–2.98 (m, 2 H), 3.32–3.36 (m, 1 H), 3.45–3.54 (m, 2 H), 6.98–7.01 (m, 1 H), 7.06–7.09 (m, 2 H), 7.31–7.33 (m, 1 H), 7.54–7.57 (m, 1 H).

***N*-(3-(4-Acetoxyphenyl)-propenoyl)-D-alanine tryptamide** was prepared from acid **6** 0.77 g (3.7 mmol) and (*R*)-**4e** 0.86 g (3.7 mmol) using ethyl chloroformate 0.36 mL (3.7 mmol) and two portions of Et_3N 1.1 mL (8.2 mmol) in THF according to the syntheses of **3a** (Sam *et al.* 1959). The carboxylic acid activation was done at 0°C. THF was evaporated and the residue was dissolved in DCM and washed four times with 30% citric acid, once with sat. NaCl aq. and three times with sat. $NaHCO_3$ aq. The organic phase was dried and the most of the DCM was evaporated. The product started to precipitate out of the solvent during the evaporation. It was filtered and washed with

DCM. The filtrate was evaporated and more product was recrystallized from DCM (40%). ^{1}H NMR (CD_3OD): δ = 1.34 (d, ^3J = 7.2 Hz, 3 H), 2.28 (s, 3 H), 2.96 (t, ^3J = 7.3 Hz, 2 H), 3.46–3.55 (m, 2 H), 4.43 (q, ^3J = 7.1 Hz, 1 H), 6.59–6.63 (m, 1 H), 6.97–7.00 (m, 1 H), 7.04–7.07 (m, 2 H), 7.13–7.15 (m, 2 H), 7.29–7.31 (m, 1 H), 7.51–7.60 (m, 4 H).

***N*-(3-(4-Hydroxyphenyl)-propenoyl)-D-alanine tryptamide ((*R*)-7e)**. *N*-(3-(4-acetoxyphenyl)-propenoyl)-D-alanine tryptamide 0.51 g (1.2 mmol) was dissolved in MeOH. The reaction flask was under argon at 0^{o}C. 2.4 mL (2.4 mmol) of 1 M CH_3ONa in MeOH was added slowly (Boiadjiev and Lightner 1996). Color of the mixture was immediately changed to yellow and the reaction was over in 15 min. Most of the MeOH was evaporated and the residue was acidified with 3 M HCl. H_2O was added drop wise to precipitate the product. The product was filtered and washed with H_2O (75%). ^{1}H NMR (CD_3OD): δ = 1.34 (d, ^3J = 7.2 Hz, 3 H), 2.96 (t, ^3J = 7.1 Hz, 2 H), 3.45-3.56 (m, 2 H), 4.42 (k, ^3J = 7.1 Hz, 1 H), 6.43–6.46 (m, 1 H), 6.79–6.81 (m, 2 H), 6.97–7.01 (m, 1 H), 7.05–7.08 (m, 2 H), 7.30–7.31 (m, 1 H), 7.41–7.43 (m, 2 H), 7.45–7.48 (m, 1 H), 7.55–7.57 (m, 1 H). ^{13}C NMR (CD_3OD): δ = 18.37, 26.13, 41.35, 50.61, 112.21, 113.06, 116.72, 118.00, 119.24, 119.61, 122.30, 123.54, 127.66, 128.70, 130.67, 138.11, 142.39, 160.59, 168.84, 175.04. ESI-MS (m/z): 378 $[M + H]^+$. Anal. ($C_{22}H_{23}N_3O_3 \cdot 0.4H_2O$) C, H, N.

Synthesis of 7f

***N*-Cbz-Glycine (2f)** was prepared from glycine 2.0 g (27 mmol) and benzyl chloroformate 9.1 g (7.6 mmol) according to the syntheses of **2b**. The product was used directly for the following coupling reaction.

***N*-Cbz-Glycine tryptamide (3f).** Compound **2f** 1.5 g (7.2 mmol), tryptamine 1.2g (7.2 mmol) and DMAP 0.09g (0.72 mmol) were dissolved in dry $CHCl_3$. After 2 min the mixture was precipitated. DCC 1.9 g (9.3 mmol) was dissolved in $CHCl_3$ and added to the reaction flask under argon at 0^{o}C (Jung and Gervay 1991). The mixture dissolved again while it was refluxed for a couple of hours. The mixture was filtered through Celite and the solvent was evaporated (18%). ^{1}H NMR (CD_3OD): δ = 2.94 (t, ^3J = 7.2 Hz, 2 H), 3.49 (t, ^3J = 7.2 Hz, 2 H), 3.73 (s, 2 H), 5.09 (s, 2 H), 6.98–7.01 (m, 1 H), 7.06–7.09 (m, 2 H), 7.29–7.35 (m, 6 H), 7.55–7.56 (m, 1 H).

Glycine tryptamide (4f) was prepared from compound **3f** 0.46 g (1.3 mmol) using 10% Pd/C 0.05 g (0.43 mmol) according to the syntheses of (*S*)-**4e** (99%). ^{1}H NMR (CD_3OD): δ = 2.96–2.99 (m, 2 H), 3.55–3.57 (m, 2 H), 3.83 (s, 2 H), 6.98–7.03 (m, 1 H), 7.06–7.09 (m, 2 H), 7.32–7.33 (m, 1 H), 7.55–7.57 (m, 1 H).

***N*-(3-(4-Acetoxyphenyl)-propenoyl)-glycine tryptamide.** Compound **6** 0.26 g (1.29 mmol), compound **4f** 0.27 g (1.29 mmol) and DMAP 0.02 g (0.16 mmol) were dissolved in dry DCM. DCC 0.35 g (1.69 mmol) was dissolved in DCM and added to the reaction flask under argon at 0°C (Jung and Gervay 1991). The reaction was proceeded only a little during 3 d. 1,4-Dioxane was added to improve the solubility. Also, more DCC 0.60 g (2.9 mmol) was added. Next day, the mixture was filtered through Celite and the solvent was evaporated. The product was purified by CombiFlash® chromatography on siliga column using manual gradient of DCM:MeOH (99:1→20:80) (23%). ^{1}H NMR ($CDCl_3$:CD_3OD): δ = 2.25 (s, 3 H), 2.91 (t, ^3J = 6.9 Hz, 2 H), 3.51 (t, ^3J = 6.9 Hz, 2 H), 3.84 (s, 2 H), 6.33–6.37 (m, 1 H), 6.98–7.10 (m, 5 H), 7.26–7.29 (m, 2 H), 7.45–7.52 (m, 4 H).

***N*-(3-(4-Hydroxyphenyl)-propenoyl)-glycine tryptamide (7f)** was prepared from *N*-(3-(4-acetoxyphenyl)-propenoyl)-glycine tryptamide 0.12 g (0.30 mmol) using 0.59 mL (0.59 mmol) of 1 M CH_3ONa in MeOH according to the syntheses of (*S*)-**7e** (82%) (Boiadjiev and Lightner 1996). ^{1}H NMR (CD_3OD): δ = 2.97 (t, ^3J = 7.2 Hz, 2 H), 3.53 (t, ^3J = 7.3 Hz, 2 H), 3.92 (s, 2 H), 6.41–6.44 (m, 1 H), 6.80–6.82 (m, 2 H), 6.99–7.02 (m, 1 H), 7.06–7.09 (m, 2 H), 7.31–7.32 (m, 1 H), 7.41–7.43 (m, 2 H), 7.47–7.50 (m, 1 H), 7.55–7.57 (m, 1 H). ^{13}C NMR (CD_3OD): δ = 25.96, 41.21, 43.70, 112.10, 112.81, 116.56, 117.64, 119.02, 119.50, 122.17, 123.34, 127.38, 128.42, 130.51, 137.83, 142.37, 160.30, 169.33, 171.28. ESI-MS (m/z): 364 $[M + H]^+$. Anal. ($C_{21}H_{21}N_3O_3$) C, H, N.

Synthesis of 7g

2-(Cbz-amino)-isobutyric acid 5-fluorotryptamide (3g) was prepared from acid **2b** 0.33 g (1.4 mmol) and 5-fluorotryptamine hydrochloride 0.50 g (2.3 mmol), which was made basic with Et_3N, using ethyl chloroformate 0.16 mL (1.7 mmol) and two portions of Et_3N 0.50 mL (3.6 mmol) in dry THF according to the syntheses of **3a**. The carboxylic acid activation was done at 0°C (Sam *et al.* 1959). The solvents were

evaporated and the residue was dissolved in DCM. The mixture was washed twice with 30% citric acid, once with sat. NaCl aq. and three times with sat. $NaHCO_3$ aq. The organic phase was dried, filtered and evaporated (60%). ^{1}H NMR (CD_3OD): δ = 1.41 (s, 6 H), 2.86–2.89 (m, 2 H), 3.44 (s, 2 H), 5.01 (s, 2 H), 6.82–6.86 (m, 1 H), 7.11 (s, 1 H), 7.20–7.33 (m, 7 H).

2-Amino-isobutyric acid 5-fluorotryptamide (4g) was prepared from compound **3g** 0.56 g (1.4 mmol) using 10% Pd/C 0.05 g (0.46 mmol) according to the syntheses of (*S*)-**4e** (92%). ^{1}H NMR (CD_3OD): δ = 1.24–1.26 (m, 6 H), 2.86–2.93 (m, 2 H), 3.45–3.48 (m, 2 H), 6.83–6.86 (m, 1 H), 7.12–7.14 (m, 1 H), 7.23–7.29 (m, 2 H).

2-(3-(4-Acetoxyphenyl)-propenoyl-amino)-isobutyric acid 5-fluorotrypt-amide was prepared from acid **6** 0.32 g (1.5 mmol) and compound **4g** 0.34 g (1.3 mmol) using ethyl chloroformate 0.12 mL (1.3 mmol) and two portions of Et_3N 0.20 mL (1.4 mmol) in THF according to the syntheses of **3a** (Furniss *et al.* 1989, Sam *et al.* 1959). The carboxylic acid activation was done at 0°C. The reaction was stirred for 3 d. The product was dissolved in $CHCl_3$/MeOH 2:1-mixture and washed as described above. The product was yet recrystallized from DCM (43%). ^{1}H NMR (CD_3OD): δ = 1.49 (s, 6 H), 2.29 (s, 3 H), 2.89–2.93 (m, 2 H), 3.46–3.52 (m, 2 H), 6.56 (d, ^3J = 15.8 Hz, 1 H), 6.79–6.86 (m, 1 H), 7.12–7.14 (m, 3 H), 7.21–7.28 (m, 2 H), 7.46 (d, ^3J = 15.8 Hz, 1 H), 7.57–7.58 (m, 2 H).

2-(3-(4-Hydroxyphenyl)-propenoyl-amino)-isobutyric acid 5-fluorotrypt-amide (7g) was prepared from 2-(3-(4-acetoxyphenyl)-propenoyl-amino)-isobutyric acid 5-fluorotryptamide 0.25 g (0.56 mmol) using 1.12 mL (1.12 mmol) of 1 M CH_3ONa in MeOH according to the syntheses of (*S*)-**7e** (95%) (Boiadjiev and Lightner 1996). ^{1}H NMR (CD_3OD): δ = 1.48 (s, 6 H), 2.91 (t, ^3J = 7.3 Hz, 2 H), 3.47 (t, ^3J = 7.3 Hz, 2 H), 6.40–6.44 (m, 1 H), 6.79–6.81 (m, 3 H), 7.12 (s, 1 H), 7.21–7.25 (m, 2 H), 7.39–7.42 (m, 3 H). ^{13}C NMR (CD_3OD): δ = 25.73, 25.92, 41.35, 57.81, 103.77, 103.96, 110.15, 110.37, 112.76, 112.83, 113.35, 116.61, 118.46, 125.48, 127.63, 130.52, 134.49, 141.90, 157.76, 159.61, 160.35, 168.39, 176.94. ESI-MS (m/z): 410 $[M + H]^+$. Anal. ($C_{23}H_{24}FN_3O_3 \cdot 0.1H_2O$) C, H, N.

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7 CHARACTERIZATION OF THE BINDING PROPERTIES OF SIRT2 INHIBITORS WITH A *N*-(3-PHENYLPROPENOYL)-GLYCINE TRYPTAMIDE BACKBONE *

Abstract: SIRT2 inhibitors with a *N*-(3-phenylpropenoyl)-glycine tryptamide backbone were studied. This backbone has been developed in our group, and it is derived from a compound originally found by virtual screening. In addition, compounds with a smaller 3-phenylpropenoic acid tryptamide backbone were also included in the study. The binding modes for the new compounds and the previously reported compounds were analyzed with molecular modelling methods. Based on the results, three binding modes A, B, and C were chosen for further characterization. The *N*-(3-phenylpropenoyl)-glycine tryptamide backbone is a good backbone for SIRT2 inhibitors, and the series of compounds includes several potent SIRT2 inhibitors.

* Adapted with permission from: Kiviranta PH,[#] Salo HS,[#] Leppänen J, Rinne VM, Kyrylenko S, Kuusisto E, Suuronen T, Salminen A, Poso A, Lahtela-Kakkonen M, Wallén EAA. Characterization of the binding properties of SIRT2 inhibitors with a *N*-(3-phenylpropenoyl)-glycine tryptamide backbone. Bioorganic & Medicinal Chemistry 16: 8054-8062, 2008. [#] Authors contributed equally to this work.

7.1 Introduction

The first SIRT2 inhibitor with a *N*-(3-phenylpropenoyl)-glycine tryptamide backbone was compound **1**, which was found by virtual screening (Tervo *et al.* 2006). The study in which the size of compound **1** was reduced resulting in a series of *N*-(3-(4-hydroxyphenyl)-propenoyl)-amino acid tryptamides was described in the previous Chapter 6. 2-(3-(4-Hydroxyphenyl)-propenoyl-amino)-isobutyric acid tryptamide **5** and *N*-(3-(4-hydroxyphenyl)-propenoyl)-L-alanine tryptamide **6** were the most potent compounds of the series (figure 7.1) (Kiviranta *et al.* 2007).

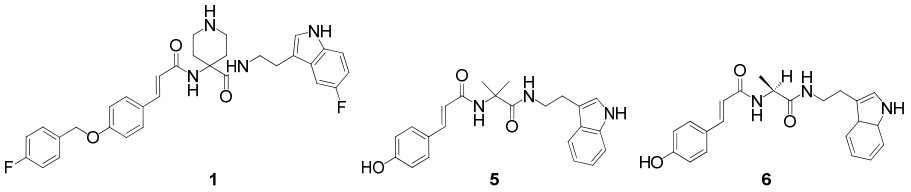


Figure 7.1. Compound **1** found by using virtual screening and its potent derivatives **5** and **6** (Kiviranta *et al.* 2007, Tervo *et al.* 2006).

In order to study the *N*-(3-phenylpropenoyl)-glycine tryptamide backbone, a new series of more modified compounds were synthesized. The modifications also included changes in the *N*-(3-phenylpropenoyl)-glycine tryptamide backbone, such as reduction of the double bond and replacing the indole ring by another aromatic ring. The new series of compounds was designed to include more variations than the earlier presented series of compounds. The study also included compounds with a shorter 3-phenylpropenoic acid tryptamide backbone.

Molecular modelling methods were used to characterize the binding and interaction properties of all compounds (also the previously presented *N*-(3-(4-hydroxyphenyl)-propenoyl)-amino acid tryptamides **1**–**9**). So far only a little is known about the binding properties of SIRT2 inhibitors. Currently there is only one experimental crystal structure of SIRT2 publicly available (Finnin *et al.* 2001). As the only published structure is in apo-form it does not provide any experimental data on the binding sites and binding modes of the small-molecule regulators. The search was concentrated on the active site and the cavity formations around the catalytic site.

7.2 Synthetic chemistry

The new compounds **10**–**27** are based on a similar chemical backbone as the earlier presented compounds **1**–**9**, and they were synthesized by similar methods as described in previous Chapter 6. A few simplified compounds without the amino acid in the middle of the backbone were also synthesized (compounds **28**–**30**). General structures of the new compounds are described in figure 7.2.

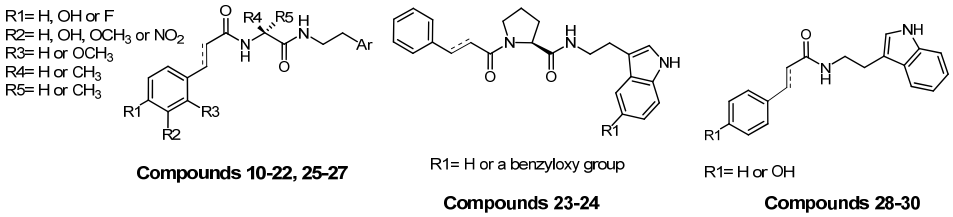


Figure 7.2. The general structures of new compounds **10**–**30**.

In amide formation reactions the carboxylic acid was activated either as an anhydride using ethyl chloroformate or pivaloyl chloride, as an acid chloride using oxalyl chloride or thionyl chloride, or as an *N*-hydroxysuccinimide ester using HOSu and DCC. The latter procedure was used for activation of the carboxylic acid terminal of L-alanine to avoid racemization (Garcia-Lopez *et al.* 1987).

7.3 Results and discussion

7.3.1 Inhibitory activities

The structures of the compounds, their inhibitory activities at 200 μM and the IC_{50} values for the most potent compounds are presented in tables 7.1 and 7.2, respectively. The inhibitory activities are compared to the results of the earlier presented compounds **1**–**9**. From the presented 21 new compounds, five compounds gave an inhibition of over 50% at 200 μM (**11**, **12**, **15**, **27**, and **29**), and their IC_{50} values were determined.

The earlier presented compounds showed that the central amino acids *α*-aminoisobutyric acid and L-alanine gave equipotent compounds, whereas the central amino acid glycine resulted in less potent compounds. All of these central amino acids were also used in the new series.

In the first group of new compounds **10–20** the substitution of phenyl ring was varied and the adjacent double bond was reduced. Compounds **11**, **12**, and **15** gave over 50% inhibition at 200 μM. Compounds **11** and **12** without the substituent on the phenyl ring gave inhibitions of 49.5% and 59.6% at 200 μM and IC_{50} values of 100 μM and 109 μM, respectively. Compound **15** with the *para*-fluoro substituent on the phenyl ring gave an inhibition of 61.3% at 200 μM and an IC_{50} of 105 μM. These compounds are not as potent as the corresponding earlier presented *para*-hydroxy substituted compounds **5** and **6**, but more potent than the corresponding *meta*-hydroxy and *meta*- or *ortho*-methoxy substituted compounds **10**, **13**, and **14**. Also a *meta*-nitro substituted compound **16** gave an equipotent inhibitory activity with compound **13**.

Replacing the central amino acid alanine by glycine gave **17** and **18**, which gave the lower inhibitory activities, 22.7% and 24.2% at 200 μM, respectively. Reduction of the adjacent double bond of the phenyl group resulted in compounds **19** and **20**. Increased flexibility decreased the inhibitory activity. These two compounds were clearly less potent than the corresponding compounds **11** and **5** with the double bond, respectively.

The second group of compounds constitute of **21** and **22** where the indole ring has been replaced by a 3-pyridyl and a phenyl ring. These compounds gave lower inhibitory activities as compared to the corresponding indole ring containing compounds **5** and **12**. The 3-pyridyl ring reduced the inhibitory activity strongly, whereas the phenyl ring only lowered the inhibitory activity slightly.

The effect of a more rigid central amino acid proline was studied with compounds **23** and **24**. Compound **23** has an intermediate inhibitory activity as compared to compounds **12** and **17** with the central amino acids L-alanine and glycine, respectively. The third group of compounds constitute of **24**–**27**, which have a benzyloxy group in 5-position of the indole ring. The benzyloxy group did not have a significant effect on the inhibitory activity. Also a similar effect was observed with a methoxy group in the 5-position of the indole ring in compound **18**. However, compound **27** with a reduced double bond in combination with a benzyloxy group in the 5-position of the indole ring gave a good inhibitory activity, with an inhibition of 50.2% at 200 μM and an IC_{50} value of 86 μM. It was even slightly more potent than the corresponding compound with a

double bond **25** and the other reduced compounds without the benzyloxy substituents **19** and **20**.

The fourth group of compounds **28**–**30** with the shorter 3-phenylpropenoic acid tryptamide backbone is presented in table 7.2. Interestingly, this group of relatively small compounds gave good inhibitory activities as compared to compounds discussed above. The best compound **29** gave an inhibition of 58.0% at 200 µM and an IC_{50} of 173 µM.

7.3.2 Binding mode prediction

The interaction possibilities between the area around the active site and the inhibitors were investigated with a molecular modelling approach, which includes a combination of molecular dynamics and molecular docking. In the molecular modelling studies performed on the most potent compound belonging to the series, compound **6**, brought out three distinct binding modes, which were used for analyzing the effects of structural modifications on the binding properties of the compounds. The binding modes are later referred as binding modes **A**, **B**, and **C** (figure 7.3). The structure-activity relationships analyses were made in the light of these three possible binding modes.

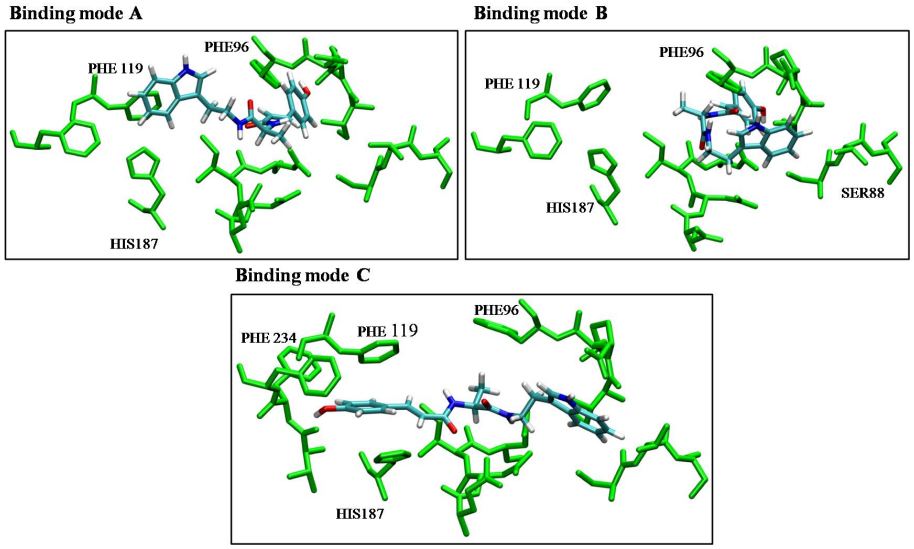
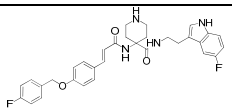
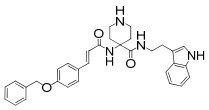
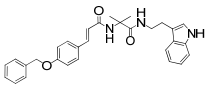
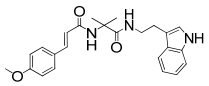
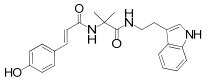
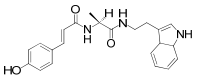
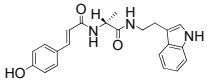
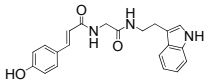
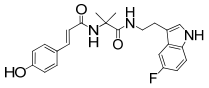
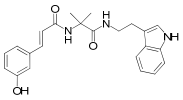
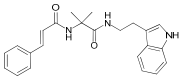
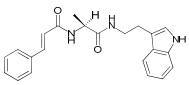
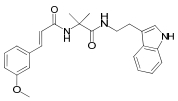
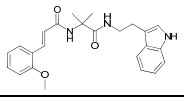
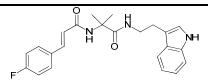
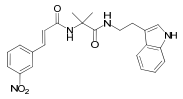
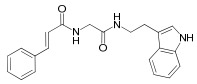
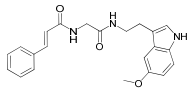
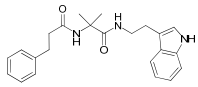
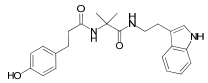
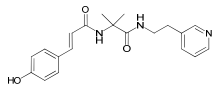
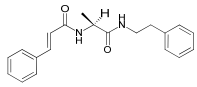
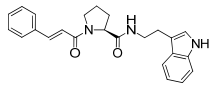
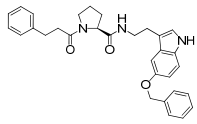
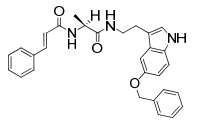
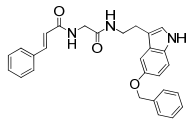
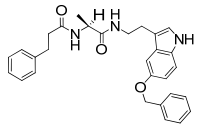


Figure 7.3. Binding modes **A**, **B**, and **C**.

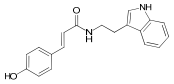
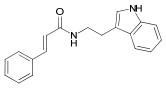
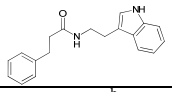
Table 7.1. SIRT2 inhibitors with *N*-(3-phenylpropenoyl)-glycine tryptamide backbone (95% confidence intervals for IC_{50} given in parentheses).

Compd	Structure	Inhibition at 200 μM ± SD, [a] %	IC_{50} (μM) [b]
1 [c]		77.3 ± 4.8	51 (27–75)
2 [c]		79.1 ± 1.4	63 (41–96)
3 [c]		32.6 ± 18.2	-
4 [c]		55.4 ± 3.3	99 (66–150)
5 [c]		83.3 ± 3.7	50 (23–109)
6 [c]		88.6 ± 0.8	47 (28–79)
7 [c]		8.5 ± 6.0	-
8 [c]		44.3 ± 10.6	-
9 [c]		67.5 ± 1.1	80 (53–120)
10		46.7 ± 0.1	-
11		49.5 ± 0.7	100 (71–141)
12		59.6 ± 11.3	109 (49–243)
13		45.9 ± 15.5	-
14		35.0 ± 8.5	-

15		61.3 ± 0.5	105 (53–209)
16		42.1 ± 15.3	-
17		22.7 ± 11.9	-
18		24.2 ± 3.7	-
19		12.5 ± 1.3	-
20		19.5 ± 1.2	-
21		2.0 ± 2.8	-
22		29.6 ± 1.8	-
23		36.6 ± 8.4	-
24		29.8 ± 3.2	-
25		43.7 ± 2.7	-
26		32.9 ± 2.3	-
27		50.2 ± 1.1	86 (34–216)

[a] SD, standard deviation.
[b] IC_{50} were determined for compounds which had over 50% inhibition at 200 μM for SIRT2.
[c] (Kiviranta *et al.* 2007)

Table 7.2. SIRT2 inhibitors with 3-phenylpropenoic acid tryptamide backbone (95% confidence intervals for IC_{50} given in parentheses)

Compd	Structure	Inhibition at 200 μM ± SD [a] %	IC_{50} (μM) [b]
28		44.3 ± 4.1	-
29		58.0 ± 11.1	173 (108–276)
30		44.1 ± 7.4	-

[a] SD, standard deviation. [b] IC_{50} were determined for compounds which had over 50% inhibition at 200 μM for SIRT2.

7.3.3 SAR

The differently substituted phenyl groups showed that a hydroxyl group in the *para*-position of the phenyl ring had a positive effect on the inhibitory activity. Compounds **5**, **6**, and **9** had an inhibition in the range 67.5–88.6% at 200 μM. In binding modes **A** and **B** hydrogen bonding possibilities for a hydroxyl group can be found at the end of the narrow channel connected to the active site of the protein. Compounds without a hydroxyl group in this position cannot form these interactions. In these binding modes any of the substituents, which compounds **10**, **13**, **14**, and **16** have in the *meta*- and *ortho*-positions, do not offer any hydrogen bond interaction possibilities. Neither does the binding mode offer the hydrogen bond possibility for compound **4** that has a methoxy substituent in *para*-position. However, compound **4** gave an inhibition of 55.4% at 200 μM and IC_{50} was 99 μM. Binding mode **C** also offers a hydrogen bond possibility for a hydroxyl group in the *para*-position. The same as in the binding modes **A** and **B**, the binding mode **C** does not offer additional hydrogen bonding interactions for structures with other substituents or substituents in other positions.

Changes made to the central amino acids located between the amide structures had various effects on the inhibitory activity. Changing the alanine residue of **6** to glycine residue of **8** or proline residue of **23** lowered the inhibition to 40% at 200 μM. However, changing the alanine residue of **6** or **12** to α-aminoisobutyric acid in **5** or **11** had no effect on the inhibitory activity. Explanations for the effect of such small changes could

not be easily found based on the binding modes. The proline residue could restrict forming of low energy conformations, which would allow favorable interactions with the protein. In the case of the glycine residue the inhibitory activity could be affected by the increased conformational flexibility. As the flexibility increases the binding process has larger negative effect on the entropic term of the binding free energy as in the case of more rigid compounds.

Compounds **11** and **12** gave higher inhibitions of 49.5% and 59.6% at 200 μM than similar compounds **21** and **22** with the indole ring replaced by other aryl groups. The inhibitory activities of **21** and **22** were 2.0% and 29.6% at 200 μM, respectively. This clearly indicates that the indole ring is forming important interactions in the binding site. The interactions of the indole ring in all of the binding modes **A**, **B**, and **C** are mainly hydrophobic and aromatic interactions. Similar interactions are also possible for compounds **21** and **22**. The larger molecular structure of the indole ring could, however, make such interactions stronger. Compound **21** was obtained low scoring values in both scoring functions and in all individual docking series. A clear difference could not be seen in the binding modes of these compounds.

Addition of a benzyloxy group to the 5-position of the indole ring of compounds **12** and **17** resulting in compounds **25** and **26** had no effect on the inhibitory activity. Compounds **12** and **25** had inhibitions in the range of 59.6–43.7% at 200 μM, and compounds **17** and **26** had inhibitions in the range 22.7–32.9% at 200 μM. As increased molecular structure offers more interactions upon binding and thus higher inhibitory activity, the fact that the inhibitory activity does not increase indicate that there are no favorable interaction partners for the benzyloxy group in the binding site.

In addition, the relatively small compounds **28–30** were almost equipotent. The inhibitory activities of **28**, **29**, and **30** were 44.3%, 58.0%, and 44.1% at 200 μM, respectively, and the IC_{50} value of **29** was 173 μM. The *para*-hydroxy substituent on phenyl ring did not seem to be as important for the inhibitory activity among these compounds as in the case of the bigger compounds. Removal of the *para*-hydroxy substituent of **28** did not lower the inhibitory activity. Neither did the removal of the double bond in the propenoyl side-chain lower the inhibitory activity. These differences could raise the question whether the binding of these smaller compounds differs from

the binding of the other compounds. Based on the molecular modelling results, the compounds **28**–**30** had docking poses resembling binding modes **B** and **C**. However, these modelling results do not explain the differences, and further studies would be needed.

Compound **28** is also a known natural product isolated from kernels of maize, safflower seed and *Ravensara anisata* Danguy, among others (Andrianaivoravelona *et al.* 1999, Sato *et al.* 1985, Takii *et al.* 2003).

7.4 Conclusions

The most potent new compounds were **11**, **12**, **15**, **27**, and **29** with IC_{50} values in the range 86–173 μM. As compared to the best earlier presented compounds with the same backbones **5** and **6** with IC_{50} values in the range 47–50 μM, these new compounds did not lead to an improvement of the inhibitory activity. However, the new compounds clearly indicate that the *N*-(3-phenylpropenoyl)-glycine tryptamide backbone is a good backbone for the design of SIRT2 inhibitors. One-third of the synthesized compounds have over 50% inhibition at the concentration of 200 μM. In addition, the study revealed that a series of compounds with a smaller 3-phenylpropenoic acid tryptamide backbone also were good SIRT2 inhibitors.

The study gave important information about how the compounds interact with SIRT2. Reasonable binding modes were found for these compounds in the area, which has been earlier postulated as a binding site for sirtuin inhibitors (Huhtiniemi *et al.* 2006, Neugebauer *et al.* 2008, Outeiro *et al.* 2007). However, prediction of a binding mode without experimental structural data is an extremely challenging task.

7.5 Synthetic procedures and analytical data

2-(Cbz-amino)-isobutyric acid (10a). 2-Amino-2-methyl-propionic acid (3.0 g, 29 mmol) was dissolved in the mixture of 1,4-dioxane:water (1:2) where 5% K_2CO_3 (0.36 M) was added. Benzyl chloroformate (8.3 mL, 58 mmol) was dissolved in the small amount of 1,4-dioxane and added to the mixture. The mixture was stirred at rt overnight. Afterwards, diethyl ether and sat. $NaHCO_3$ aq. were added. Phases were separated and the organic phase was yet extracted ones with $NaHCO_3$. The water phases

were combined and made acidic with 2 M HCl. The product was extracted with DCM, dried and evaporated. The product was used directly for the following coupling reaction. ^{1}H NMR ($(CD_3)_2CO$): δ = 1.52 (s, 6 H), 5.04 (s, 2 H), 6.54 (s, NH), 7.27–7.37 (m, 5 H), 10.91 (s, OH).

2-(Cbz-amino)-isobutyric acid tryptamide (10b). 10a (1.50 g, 6.32 mmol) and Et_3N (1.0 mL, 6.95 mmol) were dissolved in dry DCM and ethyl chloroformate (0.60 mL, 6.32 mmol) was added to active the carboxylic acid during an hour at -20°C under argon atmosphere. Another portion of Et_3N (0.88 mL, 6.32 mmol) and tryptamine (1.11 g, 6.95 mmol) were added, and the mixture was stirred under an argon atmosphere at rt overnight (Sam *et al.* 1959). The mixture was washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. Some of the product was already crystallized during citric acid wash. The organic phase was dried and evaporated. The product was recrystallized from ethyl acetate and hexane (49%). ^{1}H NMR ($CDCl_3$): δ = 1.46 (s, 6 H), 2.93–2.96 (m, 2 H), 3.56–3.60 (m, 2 H), 5.04 (s, 2 H), 6.97 (s, 1 H), 7.10–7.13 (m, 1 H), 7.18–7.21 (m, 1 H), 7.32–7.37 (m, 5 H), 7.59–7.61 (m, 1 H), 7.88 (s, 1 H).

2-Amino-isobutyric acid tryptamide (10c). 10b (1.17 g, 3.08 mmol) was dissolved in dry MeOH and ammoniumformate (0.58 g, 9.25 mmol) and 10% Pd/C (0.10 g, 0.92 mmol) were added. The mixture was stirred under an argon atmosphere at rt overnight, filtered through Celite and evaporated. The residue was dissolved in DCM, washed with sat. Na_2CO_3 aq., and the solvent was evaporated (98%). ^{1}H NMR (CD_3OD): δ = 1.25 (s, 6 H), 2.96 (t, ^3J = 7.2 Hz, 2 H), 3.49 (t, ^3J = 7.2 Hz, 2 H), 6.98–7.01 (m, 1 H), 7.06–7.10 (m, 2 H), 7.32–7.33 (m, 1 H), 7.57–7.58 (m, 1 H).

3-(3-Acetoxyphenyl)-propenoic acid (10d). 3-(3-Hydroxyphenyl)-propenoic acid (0.20 g, 1.22 mmol) was dissolved in pyridine and acetic anhydride (0.30 mL, 3.05 mmol) was added. The mixture was stirred at rt overnight. The mixture was poured into the ice-water (100 mL) and made acidic with 3 M HCl. The precipitated product was filtered and washed with water and diethyl ether (28%). ^{1}H NMR (DMSO-d_6): δ = 2.28 (s, 3 H), 6.55 (d, ^3J = 16.1 Hz, 1 H), 7.16–7.19 (m, 1 H), 7.43–7.47 (m, 1 H), 7.49–7.50 (m, 1 H), 7.56–7.59 (m, 2 H), 12.44 (s, OH).

2-(3-(3-Acetoxyphenyl)-propenoyl-amino)-isobutyric acid tryptamide (10e) was prepared from **10d** (0.06 g, 0.27 mmol) and **10c** (0.06 g, 0.26 mmol) using ethyl chloroformate (0.03 mL, 0.32 mmol) and two portions of Et_3N (0.09 mL, 0.66 mmol) in THF according to the syntheses of **10b**, except the carboxylic acid activation was done under argon atmosphere at 0°C for 1.5 h (Sam *et al.* 1959). THF was evaporated. The residue was dissolved in DCM and washed with 30% citric acid, sat. NaCl aq. and sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified by CombiFlash® chromatography on silica column (DCM:MeOH, 99:1) (69%).

2-(3-(3-Hydroxyphenyl)-propenoyl-amino)-isobutyric acid tryptamide (10). 10e (0.08 g, 0.18 mmol) was dissolved in DCM. A small amount of MeOH was added to dissolve the substance completely. The reaction flask was under argon at 0°C. 1 M CH_3ONa (0.36 mL, 0.36 mmol) in MeOH was added (Boiadjiev and Lightner 1996). Colour of the mixture was immediately changed to yellow. Another portion of 1 M CH_3ONa in MeOH was added and the ice-water bath was removed. The mixture was stirred at rt overnight. DCM was evaporated and the residue was dissolved in MeOH and acidified with 3 M HCl. Water was added dropwise to precipitate the product. The product was filtered and washed with diethyl ether and DCM (71%). ^{1}H NMR (CD_3OD): δ = 1.48 (s, 6 H), 2.96 (t, ^3J = 7.2 Hz, 2 H), 3.50 (t, ^3J = 7.3 Hz, 2 H), 6.54 (d, ^3J = 15.8 Hz, 1 H), 6.80–6.81 (m, 1 H), 6.97–7.07 (m, 5 H), 7.18–7.22 (m, 1 H), 7.28–7.30 (m, 1 H), 7.40 (d, ^3J = 15.8 Hz, 1 H), 7.58–7.59 (m, 1 H). ^{13}C NMR (CD_3OD): δ = 25.71, 26.05, 41.58, 57.94, 112.18, 113.20, 115.19, 117.90, 119.29, 119.55, 120.39, 121.87, 122.25, 123.52, 128.68, 130.84, 137.59, 138.06, 141.95, 158.88, 167.91, 176.85. ESI-MS (*m*/*z*): 392 $[M + H]^+$. Anal. ($C_{23}H_{25}N_3O_3 \cdot 0.3H_2O$): C, H, N.

2-Amino-isobutyric acid methyl ester (11a). 2-Amino-2-methyl-propionic acid (3.0 g, 29 mmol) was dissolved in MeOH. Thionylchloride (5.3 mL, 73 mmol) was dissolved in MeOH and added to the mixture through the dropping funnel at 0°C. The mixture was refluxed for 3 h, evaporated and dried in the vacuum. The product was used without further purification. ^{1}H NMR (DMSO-d_6): δ = 1.51 (s, 6 H), 3.76 (s, 3 H), 8.80 (s, NH_2).

2-(3-Phenyl-propenoic acid-amino)-isobutyric acid methyl ester (11b). The compound **11a** (1.2 g, 11 mmol) is dissolved in DCM and Et_3N (1.6 mL, 12 mmol) is

added. 3-Phenyl-propenoic acid chloride (1.8 g, 11 mmol) is partly dissolved in DCM and added slowly to the mixture. The mixture is stirred at rt for 3 d. The mixture was washed with 30% citric acid, sat. NaCl aq. and sat. $NaHCO_3$ aq., dried and evaporated. The product was purified by flash chromatography using a manual gradient of ethyl acetate:petrol ether (50:50→60:40) (44%). ^{1}H NMR ($CDCl_3$): δ = 1.64 (s, 6 H), 3.78 (s, 3 H), 6.24 (s, NH), 6.38-6.42 (m, 1 H), 7.35-7.36 (m, 3 H), 7.48-7.50 (m, 2 H), 7.58-7.61 (m, 1 H).

2-(3-Phenyl-propenoic acid-amino)-isobutyric acid (11c). The compound **11b** (1.14 g, 4.61 mmol) was dissolved in the mixture of H_2O (55 mL) and MeOH (18 mL). $LiOH·H_2O$ (1.16 g, 27.7 mmol) was added. The mixture was stirred at rt overnight. MeOH was evaporated and more H_2O was added. The mixture was washed with DCM. The H_2O phase was made acidic with 2 M HCl. The product started to precipitate out of the solution. The H_2O phase was separated and the organic phase was evaporated. The product was dried in vacuum (87%). ^{1}H NMR (DMSO-d_6): δ = 1.41 (s, 6 H), 6.66-6.69 (m, 1 H), 7.36-7.43 (m, 3 H), 7.54-7.55 (m, 2 H), 8.25 (s, 1 H), 12.15 (s, OH).

2-(3-Phenyl-propenoic acid-amino)-isobutyric acid tryptamide (11) was prepared from **11c** (0.93 g, 4.0 mmol) and tryptamine (0.64 g, 4.0 mmol) using ethyl chloroformate (0.38 mL, 4.0 mmol) and two portions of Et_3N (1.22 mL, 8.8 mmol) in DCM according to the syntheses of **10b** (Sam *et al.* 1959). The product was purified by flash chromatography using a manual gradient of DCM:MeOH (98:2→97:3) (7%). ^{1}H NMR (CD_3OD): δ = 1.48 (s, 6 H), 2.95 (t, ^3J = 7.3 Hz, 2 H), 3.50 (t, ^3J = 7.4 Hz, 2 H), 6.61 (d, ^3J = 15.8 Hz, 1 H), 6.96–6.99 (m, 1 H) 7.03–7.06 (m, 2 H), 7.27–7.29 (m, 1 H), 7.34-7.40 (m, 3 H), 7.47 (d, ^3J = 15.8 Hz, 1 H), 7.54–7.59 (m, 3 H). ^{13}C NMR (CD_3OD): δ = 25.72, 26.08, 41.63, 57.97, 112.18, 113.25, 119.32, 119.56, 122.09, 122.26, 123.54, 128.76, 128.86, 129.91, 130.81, 136.34, 138.13, 141.77, 167.87, 176.90. ESI-MS (*m*/*z*): 376 $[M + H]^+$. Anal. ($C_{23}H_{25}N_3O_2·1.1H_2O$): C, H, N.

3-Propenoic acid chloride (12a). 3-Phenylpropenoic acid (1.5 g, 10 mmol) was dissolved in dry DCM (40 mL) and oxalyl chloride (1.05 mL, 12 mmol) was added. A couple of drops DMF were added as a catalyser. The mixture was stirred at rt overnight, the solvent was evaporated, and the product was dried well in the vacuum. The product was used without further purification.

***N*-(3-Phenylpropenoyl)-L-alanine (12b).** Compound **12a** (1.7 g, 10 mmol) was dissolved in diethyl ether (20 mL) and L-alanine (0.89 g, 10 mmol) was dissolved in 1 M NaOH (40 mL). The solutions were combined and stirred at rt overnight. Next day, more H_2O and diethyl ether were added. The mixture was stirred, and the phases were separated. Water phase was made acidic and the product was extracted with DCM. The solvent was dried and evaporated. The product was used without further purification.

***N*-(3-Phenylpropenoyl)-L-alanine *N*-hydroxysuccinimide ester (12c).** Compound **12b** (0.80 g, 3.65 mmol) and *N*-hydroxysuccinimide (0.42 g, 3.65 mmol) were dissolved in dry ACN. *N*,*N*'-Dicyclohexylcarbodiimide (0.75 g, 3.65 mol) was dissolved in dry ACN and added dropwise to the reaction mixture at -20°C. The reaction was stirred at -20°C for 2.5 h and stored in a freezer overnight. The mixture was filtered and the filtrate was evaporated. The product was washed with hexane and dried in a vacuum. The product was used without further purification.

***N*-(3-Phenyl-propenoic acid)-L-alanine tryptamide (12).** Compound **12c** (1.27 g, 4.01 mmol) was dissolved in THF and tryptamine (1.29 g, 8.03 mmol) was added. The mixture was stirred at rt overnight. THF was evaporated and the residue was dissolved in DCM and washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified twice by flash chromatography using manual gradients of ethyl acetate:petrol ether (50:50→100:0) and DCM:MeOH (99:1→98:2) (20%). ^{1}H NMR (CD_3OD): δ = 1.35 (d, ^3J = 7.2 Hz, 3 H), 2.96 (t, ^3J = 7.2 Hz, 2 H), 3.46–3.55 (m, 2 H), 4.43 (k, ^3J = 7.1 Hz, 1 H), 6.65 (d, ^3J = 15.8 Hz, 1 H), 6.97–7.00 (m, 1 H), 7.04–7.07 (m, 2 H), 7.29–7.31 (m, 1 H), 7.35–7.41 (m, 3 H), 7.52–7.57 (m, 4 H). ^{13}C NMR (CD_3OD): δ = 18.37, 26.14, 41.38, 50.67, 112.22, 113.08, 119.26, 119.62, 121.52, 122.30, 123.56, 128.73, 128.90, 129.93, 130.88, 136.26, 138.14, 142.19, 168.23, 174.93. ESI-MS (*m*/*z*): 362 $[M + H]^+$. Anal. ($C_{22}H_{23}N_3O_2 \cdot 0.2H_2O$): C, H, N.

3-(3-Methoxyphenyl)-propenoic acid (13a). 3-Methoxybenzaldehyde (0.89 mL, 7.3 mmol), malonic acid (1.7 g, 16 mmol), pyridine (3.3 mL, 41 mmol) and a couple of drops piperidine were refluxed for 1 h. After cooling the flask, the mixture was poured into excess water containing enough HCl (>1.3 mL, 41 mmol) to combine with the

pyridine, filtered and washed with H_2O. The product was recrystallized from EtOH (Furniss *et al.* 1989).

2-(3-(3-Methoxyphenyl)-propenoyl-amino)-isobutyric acid methyl ester (13b). Acid **13a** (2.0 g, 11 mmol) and Et_3N (1.8 mL, 13 mmol) were dissolved in DCM. Pivaloyl chloride (1.4 mL, 11 mmol) was dissolved in DCM and added to the mixture through the dropping funnel at 0^oC. The mixture was stirred for 1 h. Another portion of Et_3N (1.8 mL, 13 mmol) was added. 2-Aminoisobutyric acid methyl ester **11a** (1.3 g, 11 mmol) was dissolved in DCM and added to the mixture at rt (Sam *et al.* 1959). The mixture was stirred for 3.5 h. The mixture was washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The solvent was dried and evaporated. The product was purified by flash chromatography using a manual gradient of DCM:MeOH (99:1→98:2) (52%). ^{1}H NMR ($CDCl_3$): δ = 1.64 (s, 6 H), 3.78 (s, 3 H), 3.82 (s, 3 H), 6.24 (s, 1 H), 6.39 (d, ^3J = 15.6 Hz, 1 H), 6.89–6.91 (m, 1 H), 7.01–7.02 (m, 1 H), 7.08–7.09 (m, 1 H), 7.56 (d, ^3J = 15.6 Hz, 1 H).

2-(3-(3-Methoxyphenyl)-propenoyl-amino)-isobutyric acid (13c) was prepared from **13b** (1.6 g, 5.9 mmol) in the mixture of MeOH (72 mL) and $LiOH·H_2O$ (1.5 g, 35 mmol) in H_2O (24 mL) according to the syntheses of **11c**. The mixture was washed twice with diethyl ether, and the H_2O phase was made acidic with 3M HCl. The product was extracted with diethyl ether. The product started to precipitate. Diethyl ether was evaporated and the residue was yet washed with cold diethyl ether (100%). ^{1}H NMR ($CDCl_3$): δ = 1.62 (s, 6 H), 3.83 (s, 3 H), 6.54 (d, ^3J = 15.7 Hz, 1 H), 6.88–6.90 (m, 1 H), 7.01–7.02 (m, 1 H), 7.08–7.09 (m, 1 H), 7.44 (s, 1 H), 7.50 (d, ^3J = 15.7 Hz, 1 H).

2-(3-(3-Methoxyphenyl)-propenoyl-amino)-isobutyric acid tryptamide (13) was prepared from 2-(3-(3-methoxyphenyl)-propenoyl-amino)-isobutyric acid (1.09 g, 4.14 mmol) and tryptamine (0.66 g, 4.14 mmol) using ethyl chloroformate (0.39 mL, 4.14 mmol) and two portions of Et_3N (1.26 mL, 9.10 mmol) in dry DCM according to the syntheses of **10b** (Sam *et al.* 1959). The product was washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. EtOH was added to dissolve the precipitated product. The phases were separated and the H_2O phase was extracted with the mixture of DCM/EtOH. The organic phase was evaporated and the product was purified by flash chromatography using a manual gradient of DCM:MeOH (99:1→97:3)

(20%). ^{1}H NMR (CD_3OD): δ = 1.48 (s, 6 H), 2.95 (t, ^{3}J = 7.3 Hz, 2 H), 3.49 (t, ^{3}J = 7.4 Hz, 2 H), 3.82–3.82 (m, 3 H), 6.58–6.61 (m, 1 H), 6.93–6.99 (m, 2 H), 7.03–7.06 (m, 2 H), 7.09 (s, 1 H), 7.12–7.14 (m, 1 H), 7.27–7.31 (m, 2 H), 7.42–7.45 (m, 1 H), 7.57–7.59 (m, 1 H). ^{13}C NMR (DMSO-d_6): δ = 25.09, 25.20, 39.85, 55.03, 56.09, 111.26, 111.85, 112.38, 115.13, 118.08, 118.24, 119.86, 120.78, 122.58, 123.25, 127.21, 129.88, 136.20, 136.45, 138.22, 159.54, 164.26, 173.73. ESI-MS (*m*/*z*): 406 $[M + H]^+$. Anal. ($C_{24}H_{27}N_3O_3 \cdot 0.3H_2O$): C, H, N.

3-(3-Methoxyphenyl)-propenoic acid (14a) was prepared from 2-methoxybenzaldehyde (1.0 g, 7.3 mmol), malonic acid (1.7 g, 16 mmol), pyridine (3.3 mL, 41 mmol) and a couple of drops piperidine according to the syntheses of **13a** (Furniss *et al.* 1989).

2-(3-(2-Methoxyphenyl)-propenoyl-amino)-isobutyric acid methyl ester (14b) was prepared from acid **14a** (2.3 g, 13 mmol) and 2-aminoisobutyric acid methyl ester **11a** (1.5 g, 13 mmol) using pivaloylchloride (1.6 mL, 13 mmol) and two portions of Et_3N (3.9 mL, 28 mmol) in DCM according to the syntheses of **13b** (Sam *et al.* 1959). The mixture was stirred at rt for 3 d. The mixture was washed three times with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The solvent was dried and evaporated. The product was purified twice by flash chromatography (DCM:MeOH, 80:20 and 99:1) (50%). ^{1}H NMR ($CDCl_3$): δ = 1.56 (s, 6 H), 3.70 (s, 3 H), 3.79 (s, 3 H), 6.23 (s, 1 H), 6.47 (d, ^{3}J = 15.8 Hz, 1 H), 6.81–6.91 (m, 2 H), 7.21–7.25 (m, 1 H), 7.37–7.39 (m, 1 H), 7.78 (d, ^{3}J = 15.8 Hz, 1 H).

2-(3-(2-Methoxyphenyl)-propenoyl-amino)-isobutyric acid (14c) was prepared from **14b** (1.9 g, 7.0 mmol) using the mixture of MeOH (84 mL) and $LiOH \cdot H_2O$ (1.8 g, 42 mmol) in H_2O (28 mL) according to the syntheses of **11c**. The mixture was washed twice with diethyl ether, and the H_2O phase was made acidic with 3 M HCl. The product started to precipitate during the diethyl ether extraction. The H_2O phase was separated and extracted once with DCM. The organic phase was evaporated, and dried well in a vacuum (92%). ^{1}H NMR ($CDCl_3$): δ = 1.60 (s, 6 H), 3.88 (s, 3 H), 6.64 (d, ^{3}J = 15.9 Hz, 1 H), 6.92–6.96 (m, 2 H), 7.30–7.33 (m, 1 H), 7.46–7.48 (m, 1 H), 7.81 (d, ^{3}J = 15.8 Hz, 1 H).

2-(3-(2-Methoxyphenyl)-propenoyl-amino)-isobutyric acid tryptamide (14) was prepared from 2-(3-(2-methoxyphenyl)-propenoyl-amino)-isobutyric acid 1.72 g (6.53 mmol) and tryptamine (1.05 g, 6.53 mmol) using ethyl chloroformate (0.62 mL, 6.53 mmol) and two portions of Et_3N (2.0 mL, 14 mmol) in dry DCM according to the syntheses of **10b**. The mixture was stirred at rt for 2 d (Sam *et al.* 1959). The mixture of EtOH/DCM (100 mL) was added to the reaction, and it was washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was evaporated, and the product was purified by flash chromatography using a manual gradient of DCM:MeOH (99:1→95:5) (35%). ^{1}H NMR (CD_3OD): δ = 1.47 (s, 6 H), 2.95 (t, ^3J = 7.3 Hz, 2 H), 3.49 (t, ^3J = 7.4 Hz, 2 H), 3.89–3.89 (m, 3 H), 6.66–6.70 (m, 1 H), 6.94–6.99 (m, 2 H), 7.01–7.07 (m, 3 H), 7.27–7.29 (m, 1 H), 7.33–7.36 (m, 1 H), 7.52–7.53 (m, 1 H), 7.57–7.59 (m, 1 H), 7.79–7.82 (m, 1 H). ^{13}C NMR (CD_3OD): δ = 25.77, 26.09, 41.60, 56.04, 57.95, 112.18, 112.40, 113.26, 119.34, 119.57, 121.75, 122.27, 122.43, 123.58, 124.99, 128.74, 129.47, 132.15, 137.24, 138.12, 159.65, 168.48, 176.97. ESI-MS (*m*/*z*): 406 $[M + H]^+$. Anal. ($C_{24}H_{27}N_3O_3 \cdot 0.2H_2O$): C, H, N.

2-(3-(4-Fluorophenyl)-propenoyl-amino)-isobutyric acid tryptamide (15) was prepared from 3-(4-fluoro)-propenoic acid **(**0.20 g, 1.22 mmol) and **10c** (0.30 g, 1.22 mmol) using ethyl chloroformate (0.12 mL, 1.22 mmol) and two portions of Et_3N (0.37 mL, 2.69 mmol) in THF according to the syntheses of **10b**, except the carboxylic acid activation was done at 0°C for 1 h. The mixture was stirred under an argon atmosphere at rt for 3 d (Sam *et al.* 1959). THF was evaporated. The residue was dissolved in DCM and washed with 30% citric acid, sat. NaCl aq. and sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified by recrystallization from DCM and by CombiFlash® chromatography on silica column (DCM:MeOH, 98:2) (17%). ^{1}H NMR (CD_3OD): δ = 1.48 (s, 6 H), 2.95 (t, ^3J = 7.2 Hz, 2 H), 3.50 (t, ^3J = 7.3 Hz, 2 H), 6.53 (d, ^3J = 15.8 Hz, 1 H), 6.97–7.00 (m, 1 H), 7.04–7.07 (m, 2 H), 7.10–7.13 (m, 2 H), 7.27–7.29 (m, 1 H), 7.44 (d, ^3J = 15.8 Hz, 1 H), 7.56–7.59 (m, 3 H). ^{13}C NMR (CD_3OD): δ = 25.72, 26.07, 41.61, 57.98, 112.19, 113.22, 116.78 (d, $^2J_{CF}$ = 22.10 Hz), 119.32, 119.57, 121.95, 121.96, 122.27, 123.55, 128.74, 130.89 (d, $^3J_{CF}$ = 8.44 Hz), 132.76 (d, $^4J_{CF}$ = 3.35 Hz), 138.11, 140.46, 164.98 (d, $^1J_{CF}$ = 248.79 Hz), 167.71, 176.85. ESI-MS (*m*/*z*): 394 $[M + H]^+$. Anal. ($C_{23}H_{24}FN_3O_2 \cdot 0.1H_2O$): C, H, N.

3-(3-Nitrophenyl)-propenoic acid (16a) was prepared from 3-nitrobenzaldehyde (1.0 g, 6.6 mmol), malonic acid (1.5 g, 15 mmol), pyridine (3.0 mL, 37 mmol) and a couple of drops piperidine according to the syntheses of **13** (Furniss *et al.* 1989).

2-(3-(3-Nitrophenyl)-propenoyl-amino)-isobutyric acid methyl ester (16b) was prepared from acid **16a** (1.3 g, 6.9 mmol) and 2-aminoisobutyric acid methyl ester **11a** (0.84 g, 6.9 mmol) using pivaloylchloride (0.86 mL, 6.9 mmol) and two portions of Et_3N (2.1 mL, 15 mmol) in DCM according to the syntheses of **13b** (Sam *et al.* 1959). The mixture was stirred at rt for 2 d, washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The solvent was dried and evaporated. The product was purified twice by flash chromatography using a manual gradient of ethyl acetate:petrol ether (50:50→100:0) (64%). ^{1}H NMR ($CDCl_3$): δ = 1.66 (s, 6 H), 3.79 (s, 3 H), 6.35 (s, 1 H), 6.54 (d, ^3J = 15.9 Hz, 1 H), 7.54–7.57 (m, 1 H), 7.64 (d, ^3J = 15.9 Hz, 1 H), 7.76–7.77 (m, 1 H), 8.19–8.20 (m, 1 H), 8.37 (s, 1 H).

2-(3-(3-Nitrophenyl)-propenoyl-amino)-isobutyric acid (16c) was prepared from **16b** (1.29 g, 4.41 mmol) using the mixture of MeOH (53 mL) and $LiOH·H_2O$ (1.11 g, 27 mmol) in H_2O (18 mL) according to the syntheses of **11c**. The mixture was washed with DCM, and the H_2O phase was made acidic with 2 M HCl. The product precipitated in the extraction funnel. The H_2O phase was separated, and the product was filtered and dried well in a vacuum. The H_2O phase and the filtrate contained yet some product that was extracted with the mixture of DCM:THF (4:1) (overall 97%). ^{1}H NMR ($CDCl_3$): δ = 1.43 (s, 6 H), 6.89 (d, ^3J = 15.9 Hz, 1 H), 7.50 (d, ^3J = 15.9 Hz, 1 H), 7.69–7.73 (m, 1 H), 7.99–8.01 (m, 1 H), 8.20–8.21 (m, 1 H), 8.34–8.39 (m, 1 H), 12.22 (s, OH).

2-(3-(3-Nitrophenyl)-propenoyl-amino)-isobutyric acid tryptamide (16) was prepared from 2-(3-(3-nitrophenyl)-propenoyl-amino)-isobutyric acid (0.89 g, 3.20 mmol) and tryptamine (0.51 g, 3.20 mmol) using ethyl chloroformate (0.31 mL, 3.20 mmol) and two portions of Et_3N (0.98 mL, 7.04 mmol) in the mixture of dry DCM and THF according to the syntheses of **10b** (Sam *et al.* 1959). The product was purified by flash chromatography using a manual gradient of DCM:MeOH (98:2→97:3) (7%). ^{1}H NMR (CD_3OD): δ = 1.49 (s, 6 H), 2.95 (t, ^3J = 7.2 Hz, 2 H), 3.50 (t, ^3J = 7.3 Hz, 2 H), 6.74 (d, ^3J = 15.8 Hz, 1 H), 6.96–6.99 (m, 1 H), 7.03–7.07 (m, 2 H), 7.26–7.27 (m, 1 H), 7.51 (d, ^3J = 15.8 Hz, 1 H), 7.57–7.59 (m, 1 H), 7.63–7.66 (m, 1 H), 7.91–7.93 (m, 1 H),

8.21–8.23 (m, 1 H), 8.41–8.42 (m, 1 H). ^{13}C NMR (DMSO-d_6): δ = 25.02, 25.10, 39.83, 56.13, 111.22, 111.77, 118.04, 118.19, 120.73, 121.24, 122.58, 123.49, 125.86, 127.17, 130.40, 133.83, 135.82, 136.15, 136.90, 148.22, 163.62, 173.54. ESI-MS (*m*/*z*): 421 [M + H]$^+$. Anal. ($C_{23}H_{24}N_4O_4 \cdot 0.1H_2O$): C, H, N.

***N*-(3-Phenylpropenoyl)-glycine (17a)** was prepared from 3-phenyl propenoic acid chloride (1.67 g, 10.0 mmol) in diethyl ether and glysine (0.75 g, 10.0 mmol) in 1 M NaOH (40 mL) according to the syntheses of **12b**. The H_2O phase was made acidic. The precipitated product was extracted with the DCM/EtOH mixture. The organic phase was dried and evaporated (87%). The product was used without further purification.

***N*-(3-Phenylpropenoyl)-glycine tryptamide (17)** was prepared from **17a** (0.89 g, 4.34 mmol) and tryptamine (0.70 g, 4.34 mmol) using pivaloyl chloride (0.53 mL, 4.34 mmol) and two portions of Et_3N (1.33 mL, 9.54 mmol) in DCM according to the syntheses of **13b**. The mixture was stirred at rt for 3 d (Sam *et al.* 1959). The mixture was washed twice with 30% citric acid, ones with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. A small amount of EtOH was added to dissolve the formed precipitate. The organic phase was dried and evaporated. The product was purified by flash chromatography using a manual gradient of ethyl acetate:petrol ether:MeOH (66.5:33.5:0→100:0:0→90:0:10→0:0:100) (39%). ^{1}H NMR (CD_3OD): δ = 2.96 (t, ^3J = 7.2 Hz, 2 H), 3.52 (t, ^3J = 7.3 Hz, 2 H), 3.94 (s, 2 H), 6.62–6.65 (m, 1 H), 6.98–7.01 (m, 1 H), 7.05–7.08 (m, 2 H), 7.30–7.31 (m, 1 H), 7.35–7.41 (m, 3 H), 7.54–7.58 (m, 4 H). ^{13}C NMR (CD_3OD): δ = 26.14, 41.42, 43.78, 112.20, 113.05, 119.18, 119.61, 121.38, 122.28, 123.47, 128.69, 128.87, 129.90, 130.88, 136.16, 138.09, 142.25, 168.93, 171.39. ESI-MS (*m*/*z*): 348 [M + H]$^+$. Anal. ($C_{21}H_{21}N_3O_2 \cdot 0.2H_2O$): C, H, N.

***N*-(3-Phenylpropenoyl)-glycine-5-methoxytryptamide (18)** was prepared from **17a** (0.27 g, 1.32 mmol) and 5-methoxytryptamine (0.25 g, 1.32 mmol) using pivaloyl chloride (0.16 mL, 1.32 mmol) and two portions of Et_3N (0.40 mL, 2.89 mmol) according to the syntheses of **13b**. The mixture was stirred at rt for 3 h (Sam *et al.* 1959). The mixture was washed twice with 30% citric acid, ones with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified by flash chromatography using a manual gradient of ethyl acetate:petrol ether:MeOH (75:25:0→100:0:0→99:0:1 (36%). ^{1}H NMR (CD_3OD): δ = 2.93 (t, ^3J =

7.3 Hz, 2 H), 3.51 (t, ^3J = 7.3 Hz, 2 H), 3.82 (s, 3 H), 3.94 (s, 2 H), 6.63–6.66 (m, 1 H), 6.72–6.74 (m, 1 H), 7.05–7.06 (m, 2 H), 7.18–7.20 (m, 1 H), 7.35–7.41 (m, 3 H), 7.54–7.57 (m, 3 H). ^{13}C NMR (CD_3OD): δ = 26.21, 41.32, 43.84, 56.37, 101.27, 112.61, 112.84, 112.91, 121.39, 124.29, 128.91, 129.01, 129.93, 130.92, 133.38, 136.20, 142.29, 154.96, 168.97, 171.45. ESI-MS (*m*/*z*): 378 $[M + H]^+$. Anal. ($C_{22}H_{23}N_3O_3 \cdot 0.1H_2O$): C, H, N.

2-(3-Phenyl-propanoyl-amino)-isobutyric acid methyl ester (19a). 3-Phenylpropionic acid (2.0 g, 13 mmol) was dissolved in thionyl chloride (3.0 mL, 40 mmol) and the mixture was refluxed for 1.5 h. Thionyl chloride was evaporated and the product was dried in a vacuum. 3-Phenylpropionic acid chloride (2.2 g, 13 mmol) was used in the reaction with 2-aminoisobutyric acid methyl ester **11a** (2.1 g, 18 mmol). The reaction was prepared using Et_3N (2.0 mL, 15 mmol) according to the syntheses of **11b**. The mixture was stirred at rt overnight. The product was washed three times with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified by flash chromatography using a manual gradient of ethyl acetate:petrol ether (50:50→40:60) (56%). ^{1}H NMR ($CDCl_3$): δ = 1.50 (s, 6 H), 2.47 (t, ^3J = 7.7 Hz, 2 H), 2.95 (t, ^3J = 7.7 Hz, 2 H), 3.72 (s, 3 H), 5.89 (s, 1 H), 7.18–7.21 (m, 3 H), 7.26–7.29 (m, 2 H).

2-(3-Phenyl-propanoyl-amino)-isobutyric acid (19b) was prepared from **19a** (1.9 g, 7.5 mmol) using the mixture of MeOH (90 mL) and $LiOH \cdot H_2O$ (1.9 g, 45 mmol) in H_2O (30 mL) according to the syntheses of **11c**. The mixture was washed with DCM, and the H_2O phase was made acidic with 2 M HCl. The precipitated product was extracted with DCM (the product not fully soluble) and the solvent was evaporated (97%). ^{1}H NMR ($CDCl_3$): δ = 1.49 (s, 6 H), 2.53 (t, ^3J = 7.5 Hz, 2 H), 2.97 (t, ^3J = 7.5 Hz, 2 H), 5.75 (s, 1 H), 7.19–7.22 (m, 3 H), 7.26–7.31 (m, 2 H).

2-(3-Phenyl-propanoyl-amino)-isobutyric acid tryptamide (19) was prepared from 2-(3-phenyl)-propenoyl-amino)-isobutyric acid (0.80 g, 3.40 mmol) and tryptamine (0.62 g, 3.74 mmol) using ethyl chloroformate 0.36 mL (3.40 mmol) and two portions of Et_3N (1.04 mL, 7.48 mmol) in the mixture of dry THF according to the syntheses of **10b** (Sam *et al.* 1959). DCM was added to the mixture. The product was washed 30% citric acid, sat. NaCl aq. and sat. $NaHCO_3$ aq. The organic phase was dried and

evaporated. The product was purified by flash chromatography using a manual gradient of DCM:MeOH (98:2→97:3) (17%). ^{1}H NMR (CD_3OD): δ = 1.34 (s, 6 H), 2.41–2.45 (m, 2 H), 2.82–2.85 (m, 2 H), 2.88–2.91 (m, 2 H), 3.41–3.45 (m, 2 H), 6.99–7.02 (m, 1 H), 7.06–7.09 (m, 2 H), 7.12–7.15 (m, 1 H), 7.17–7.19 (m, 2 H), 7.21–7.25 (m, 2 H), 7.31–7.32 (m, 1 H), 7.57–7.58 (m, 1 H). ^{13}C NMR (CD_3OD): δ = 25.61, 26.06, 32.52, 38.71, 41.50, 57.73, 112.24, 113.21, 119.35, 119.59, 122.32, 123.55, 127.20, 128.76, 129.42, 129.45, 138.15, 142.14, 174.59, 176.91. ESI-MS (*m*/*z*): 378 $[M + H]^+$. Anal. ($C_{23}H_{27}N_3O_2$): C, H, N.

2-Amino-isobutyric acid tryptamide (20a). Compound **10b** (2.47 g, 6.51 mmol) was dissolved in dry MeOH and 10% Pd/C (0.23 g, 2.15 mmol) was added. Hydrogen gas was led into the reaction flask from the hydrogen balloon at rt for 1 h. The mixture was filtered through Celite and the solvent was evaporated. The residue was dissolved in DCM and washed with $NaCO_3$. The product was extracted back with DCM. The organic phase was dried and evaporated (75%). 1H NMR (CD_3OD): δ = 1.25 (s, 6 H), 2.94-2.97 (m, 2 H), 3.48-3.50 (m, 2 H), 6.98-7.02 (m, 1 H), 7.07-7.10 (m, 2 H), 7.32-7.33 (m, 1 H), 7.57-7.58 (m, 1 H).

3-(4-Acetoxyphenyl)-propionic acid (20b). 3-(4-Hydroxyphenyl)-propionic acid (0.50 g, 3.0 mmol), acetic anhydride (1.42 ml, 15.0 mmol) and DMAP (73.30 mg, 0.6 mmol) were dissolved in pyridine (~ 3 ml) and stirred at rt overnight. Diethyl ether was added, and the mixture was washed with 2 M HCl and NaCl. The solvents were evaporated (Yang *et al.* 2000). The product was used directly for the following coupling reaction.

2-(3-(4-Acetoxyphenyl)-propanoyl-amino)-isobutyric acid tryptamide (20c) was prepared from the acid **20b** (0.84 g, 4.03 mmol) and **20a** (1.19 g, 4.84 mmol) using ethyl chloroformate (0.38 ml, 4.03 mmol) and two portions of Et_3N (1.24 ml, 8.88 mmol) in THF according to the syntheses of **10b**. A small amount of DCM was used to dissolve **20b** better. The carboxylic acid activation was done under argon atmosphere at 0^{o}C for 1h (Sam *et al.* 1959). THF was evaporated. The residue was dissolved in DCM and washed with 30% citric acid, sat. NaCl aq. and sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified by flash chromatography (ethyl acetate:petrol ether, 70:30) (27%). ^{1}H NMR (CD_3OD): δ = 1.35 (s, 6 H), 2.24 (s, 3 H),

2.41-2.44 (m, 2 H), 2.81-2.92 (m, 4 H), 3.42-3.46 (m, 2 H), 6.86-7.32 (m, 8 H), 7.57-7.59 (m, 1 H).

2-(3-(4-Hydroxyphenyl)-propanoyl-amino)-isobutyric acid tryptamide (20) was prepared from **20c** (0.56 g, 1.29 mmol) in DCM using 1 M CH_3ONa in MeOH (2.57 mmol) according to the syntheses of **10** (Boiadjiev and Lightner 1996). The mixture was stirred on the ice-water bath for 1 h, until the color of the mixture was changed. DCM was evaporated and the residue was dissolved in MeOH and acidified with 3 M HCl. The product did not precipitate when H_2O was added. The product was purified by flash chromatography using a manual gradient of ethyl acetate:petrol ether (70:30→100:0) (80%). ^{1}H NMR (CD_3OD): δ = 1.34 (s, 6 H), 2.39 (t, ^3J = 7.6 Hz, 2 H), 2.75 (t, ^3J = 7.6 Hz, 2 H), 2.90 (t, ^3J = 7.3 Hz, 2 H), 3.42-3.46 (m, 2H), 6.69-6.71 (m, 2 H), 6.98-7.02 (m, 3 H), 7.06-7.09 (m, 2 H), 7.17-7.19 (m, NH), 7.30-7.32 (m, 1 H), 7.57-7.59 (m, 1 H). ^{13}C NMR (CD_3OD): δ = 25.62, 26.06, 31.74, 39.04, 41.49,* 41.61,* 57.71,* 57.73,* 112.20, 113.18, 116.17, 119.35, 119.58, 122.29, 123.56, 128.75, 130.40, 132.83, 138.10, 156.73, 174.81, 176.91,* 176.99* (*) Due to the conformational effect these peaks are duplets.). ESI-MS (*m*/*z*): 394 $[M + H]^+$. Anal. ($C_{23}H_{27}N_3O_3 \cdot 0.2H_2O$): C, H, N.

2-(Cbz-amino)-isobutyric acid 2-pyridinamide (21a) was prepared from **10a** (2.30 g, 9.69 mmol) and 3-(2-aminoethyl) pyridine (0.32 g, 2.64 mmol) (the amount of the reagent left) in THF using ethyl chloroformate (0.92 mL, 9.69 mmol) and two portions of Et_3N (2.98 mL, 21.4 mmol) overnight according to the syntheses of **10b**, except the carboxylic acid activation was at 0°C under argon atmosphere (Sam *et al.* 1959). THF was evaporated and the residue was dissolved in DCM. The mixture was washed with H_2O. Some of the product was already crystallized during H_2O wash. The H_2O phase was yet extracted ones with DCM. The organic phases were combined and evaporated. The product was purified by chromatography using ethyl acetate:MeOH (90:10) (20%). ^{1}H NMR (MeOD): δ = 1.38 (s, 6 H), 2.80-2.84 (m, 2 H), 3.42 (s, 2 H), 5.04 (s, 2 H), 7.28-7.34 (m, 6 H), 7.72-7.73 (m, 1 H), 8.36-8.40 (m, 2 H).

2-Amino-isobutyric acid 2-pyridinamide (21b) was prepared from compound **21a** (0.66 g, 1.93 mmol) in MeOH using 10% Pd/C (0.07 g, 0.64 mmol) and hydrogen gas at rt for 2 h according to the syntheses of **20a** (97%). ^{1}H NMR (CD_3OD): δ = 1.24 (s, 6 H),

2.86 (t, ^3J = 7.1 Hz, 2 H), 3.45 (t, ^3J = 7.1 Hz, 2 H), 7.35-7.38 (m, 1 H), 7.73-7.74 (m, 1 H), 8.38-8.41 (m, 2 H).

3-(4-Acetoxyphenyl)-propenoic acid (21c) was prepared from 3-(4-hydroxyphenyl)-propenoic acid (13 g, 80 mmol) and acetic anhydride (19 mL, 200 mmol) in pyridine according to the syntheses of **10d** (86%). ^{1}H NMR (CD_3OD): δ = 2.28 (s, 3 H), 6.44–6.48 (m, 1 H), 7.16–7.18 (m, 2 H), 7.65–7.68 (m, 3 H).

2-(3-(4-Acetoxyphenyl)-propenoyl-amino)-isobutyric acid 2-pyridinamide (21d) was prepared from the acid **21c** (0.39 g, 1.88 mmol) and **21b** (0.39 g, 1.88 mmol) using ethyl chloroformate (0.19 ml, 1.88 mmol) and two portions of Et_3N (0.58 ml, 4.14 mmol) in THF according to the syntheses of **10b**, except the carboxylic acid activation was done under argon atmosphere at 0^{o}C for 1 h (Sam *et al.* 1959). THF was evaporated. The residue was dissolved in DCM and washed twice with 30% citric acid, ones with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The product was extracted to the H_2O phase. The product was tried to salt out from the water phase. Extractions with $CHCl_3$:EtOH (3:1) and MeOH gave the mixture of the product and salts. The product was purified by flash chromatography using ethyl acetate:MeOH (90:10) (60%). ^{1}H NMR (CD_3OD): δ = 1.45 (s, 6 H), 1.99 (s, 3 H), 2.84-2.86 (m, 2 H), 3.42-3.46 (m, 2 H), 6.42-6.45 (m, 1 H), 6.79-6.80 (m, 2 H), 7.31-7.33 (m, 1 H), 7.40-7.46 (m, 3 H), 7.75-7.76 (m, 1 H), 8.33-8.34 (m, 1 H), 8.41 (s, 1 H).

2-(3-(4-Hydroxyphenyl)-propenoyl-amino)-isobutyric acid 2-pyridinamide (21) was prepared from **21c** (0.45 g, 1.14 mmol) in DCM (and a small amount of MeOH dissolve **21c** completely) using 1 M CH_3ONa in MeOH (2.28 mmol) according to the syntheses of **10** (Boiadjiev and Lightner 1996). The mixture was stirred on the ice-water bath for 40 min. DCM was evaporated and the residue was dissolved in MeOH and acidified with 3 M HCl. The product did not precipitate when H_2O was added. The product was purified by flash chromatography using DCM:MeOH (90:10) (83%). ^{1}H NMR (CD_3OD): δ = 1.46 (s, 6 H), 2.85 (t, ^3J = 7.0 Hz, 2 H), 3.44 (t, ^3J = 7.0 Hz, 2 H), 6.43-6.46 (m, 1 H), 6.79-6.81 (m, 2 H), 7.30-7.34 (m, 1 H), 7.40-7.50 (m, 3 H), 7.73-7.75 (m, 1 H), 8.32-8.34 (m, 1 H), 8.40-8.41 (m, 1 H). ^{13}C NMR (CD_3OD): δ = 25.75, 33.47, 41.57, 57.76, 116.70, 118.50, 125.04, 127.62, 130.55, 137.15, 138.86, 141.99,

147.77, 150.43, 160.48, 168.37, 177.16. ESI-MS (*m*/*z*): 354 $[M + H]^+$. Anal. ($C_{20}H_{23}N_3O_3 \cdot 0.5H_2O$): C, H, N.

***N*-(3-Phenylpropenoyl)-L-alanine phenethylamide (22).** Compound **12c** (0.53 g, 1.68 mmol) was dissolved in dry THF. Et_3N (0.23 mL, 1.68 mmol) and 2-phenylethylamine (0.21 mL, 1.68 mmol) were added. The reaction was stirred at rt overnight. THF was evaporated and the residue was dissolved in DCM and washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified three times by chromatography using manual gradients of petrol ether:ethyl acetate (60:40→0:100) and DCM:MeOH (95.5:0.5→98:2 and 99:1) (16%). ^{1}H NMR (CD_3OD): δ = 1.33–1.34 (m, 3 H), 2.80 (t, ^3J = 7.2 Hz, 2 H), 3.36–3.49 (m, 2 H), 4.39–4.43 (m, 1 H), 6.64–6.68 (m, 1 H), 7.14–7.17 (m, 1 H), 7.20–7.27 (m, 4 H), 7.35–7.41 (m, 3 H), 7.53–7.57 (m, 3 H). ^{13}C NMR (CD_3OD): δ = 18.32, 36.50, 42.00, 50.69, 121.55, 127.35, 128.90, 129.51, 129.92, 129.99, 130.94, 136.29, 140.46, 142.25. ESI-MS (*m*/*z*): 323 $[M + H]^+$. Anal. ($C_{20}H_{22}N_2O_2 \cdot 0.02H_2O$): C, H, N.

***N*-(3-Phenylpropenoyl)-L-proline (23a)** was prepared from **12a** (1.67 g, 10.0 mmol) in diethyl ether (20 mL) and L-proline (1.15 g, 10.0 mmol) in 1 M NaOH (40 mL) according to the syntheses of **12b**. The H_2O phase was made acidic. The product was extracted several times with diethyl ether. The product started to precipitate in the extraction funnel. DCM and EtOH were added to dissolve the product. The organic phase was dried and evaporated (93%). ^{1}H NMR ($CDCl_3$): δ = 1.98–2.17 (m, 3 H), 2.51–2.55 (m, 1 H), 3.66–3.71 (m, 1 H), 3.77–3.81 (m, 1 H), 4.73–4.75 (m, 1 H), 6.72 (d, ^3J = 15.5 Hz, 1 H), 7.39–7.40 (m, 3 H), 7.54–7.56 (m, 2 H), 7.82 (d, ^3J = 15.4 Hz, 1 H), 9.99 (s, OH).

***N*-(3-Phenylpropenoyl)-L-proline tryptamide (23)** was prepared from **23a** (1.0 g, 4.08 mmol) and tryptamine (0.65 g, 4.08 mmol) using pivaloyl chloride (0.50 mL, 4.08 mmol) and two portions of Et_3N (1.26 mL, 8.96 mmol) according to the syntheses of **13b**, except the carboxylic acid activation was done at 0°C for 2 h. After the addition of tryptamine, the mixture was stirred at rt for a couple of hours and stored in a refrigerator overnight (Sam *et al.* 1959). The mixture was washed twice with 30% citric acid, ones with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was dried and

evaporated. The product was purified by flash chromatography using ethyl acetate (91%). ^{1}H NMR (CD_3OD): δ (major, 70%) = 1.80–2.28 (m, 4 H), 2.96 (t, ^{3}J = 7.2 Hz, 2 H), 3.49–3.77 (m, 4 H), 4.44–4.46 (m, 1 H), 6.85–6.88 (m, 1 H), 6.95–7.07 (m, 3 H), 7.29–7.59 (m, 8 H). ^{1}H NMR (CD_3OD): δ (minor, 30%) = 1.80–2.28 (m, 4 H), 2.91 (t, ^{3}J = 7.2 Hz, 2 H), 3.49–3.77 (m, 4 H), 4.51–4.53 (m, 1 H), 6.59–6.62 (m, 1 H), 6.95–7.07 (m, 3 H), 7.29–7.59 (m, 8 H). ^{13}C NMR (CD_3OD): δ (major, 70%) = 25.55, 25.99, 30.65, 41.31, 48.60, 61.93, 112.21, 113.05, 119.22, 119.45, 119.57, 122.25, 123.57, 128.72, 129.06, 129.87, 130.99, 136.24, 138.01, 143.68, 167.36, 174.31. ^{13}C NMR (CD_3OD): δ (minor, 30%) = 23.63, 26.23, 33.02, 41.09, 48.25, 62.15, 112.25, 112.81, 119.17, 119.45, 119.58, 122.34, 123.23, 128.59, 128.90, 129.90, 131.03, 136.06, 138.03, 143.73, 167.61, 174.55. ESI-MS (*m*/*z*): 388 $[M + H]^{+}$. Anal. ($C_{24}H_{25}N_3O_2$·0.3EtOAc): C, H, N.

***N*-(3-Phenylpropanoyl)-L-proline (24a).** 3-Phenyl propanoic acid (0.75 g, 5.00 mmol) was dissolved in 1.1 mL (15.0 mmol) of thionyl chloride. The mixture was refluxed gently for 1.5 h, and the solvents were evaporated. *N*-(3-Phenylpropanoyl)-L-proline was prepared from 3-phenyl propanoic acid chloride in diethyl ether and L-proline 0.58 g (5.00 mmol) in 1 M NaOH (40 mL) according to the syntheses of **12b**. The H_2O phase was made acidic with 2 M HCl and the product was extracted with DCM. The organic phase was dried and evaporated. ^{1}H NMR ($CDCl_3$): δ = 1.86–2.02 (m, 3 H), 2.44–2.52 (m, 1 H), 2.66–2.70 (m, 2 H), 2.95–3.03 (m, 2 H), 3.27–3.32 (m, 1 H), 3.38–3.42 (m, 1 H), 4.59–4.61 (m, 1 H), 7.20–7.23 (m, 3 H), 7.26–7.31 (m, 2 H).

***N*-(3-Phenylpropanoyl)-L-proline-5-benzyloxytryptamide (24)** was prepared from **24a** (0.41 g, 1.65 mmol) and 5-benzyloxytryptamine (0.50 g, 1.65 mmol) using pivaloyl chloride (0.20 mL, 1.65 mmol) and two portions of Et_3N (0.51 mL, 3.64 mmol) in DCM according to the syntheses of **13b**. The mixture was stirred at rt overnight (Sam *et al.* 1959). The mixture was washed twice with 30% citric acid, ones with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified by flash chromatography using ethyl acetate (64%). ^{1}H NMR (CD_3OD): δ (major, 70%) = 1.72–2.09 (m, 4 H), 2.55–2.65 (m, 2 H), 2.74–2.92 (m, 4 H), 3.40–3.55 (m, 4 H), 4.32–4.34 (m, 1 H), 5.09 (s, 2 H), 6.83–6.86 (m, 1 H), 7.06 (s, 1 H), 7.09–7.47 (m, 12 H). ^{1}H NMR (CD_3OD): δ (minor, 30%) = 1.72–2.09 (m, 4 H), 2.23–2.40 (m, 2

H), 2.74–2.92 (m, 4 H), 3.40–3.55 (m, 4 H), 4.07–4.09 (m, 1 H), 5.08 (s, 2 H), 6.83–6.86 (m, 1 H), 6.97 (s, 1 H), 7.09–7.47 (m, 12 H). ^{13}C NMR (CD_3OD): δ (major, 70%) = 25.45, 26.02, 30.64, 31.89, 37.27, 41.18, 48.57, 61.55, 72.16, 103.32, 112.88, 112.89, 113.23, 124.52, 127.15, 128.69, 129.08, 129.36, 129.39, 129.43, 133.55, 139.32, 142.30, 153.97, 173.95, 174.41. ^{13}C NMR (CD_3OD): δ (minor, 30%) = 23.52, 26.03, 32.14, 32.97, 37.14, 40.92, 47.90, 62.16, 72.12, 103.28, 112.60, 112.95, 113.33, 124.35, 127.16, 128.65, 129.04, 129.33, 129.36, 129.45, 133.57, 139.30, 142.17, 153.99, 174.05, 174.24. ESI-MS (*m*/*z*): 495 $[M + H]^+$ and 518 $[M + Na]^+$. Anal. ($C_{31}H_{33}N_3O_3$): C, H, N.

***N*-(3-Phenylpropenoyl)-L-alanine-5-benzyloxytryptamide (25).** Compound **12c** (0.50 g, 1.58 mmol) was dissolved in dry THF. Et_3N (0.22 mL, 1.58 mmol) and 5-benzyloxytryptamine (0.48 g, 1.58 mmol) were added. The mixture was stirred at rt overnight. THF was evaporated and the residue was dissolved in DCM and a small amount of EtOH. The product was washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified three times by flash chromatography using manual gradients of ethyl acetate:petrol ether (75:25 and 40:60→100:0) and DCM:MeOH (99:1) (22%). ^{1}H NMR (CD_3OD): δ = 1.34–1.36 (m, 3 H), 2.91 (t, ^3J = 7.3 Hz, 2 H), 3.44–3.53 (m, 2 H), 4.40–4.45 (m, 1 H), 5.09 (m, 2 H), 6.62–6.66 (m, 1 H), 6.80–6.82 (m, 1 H), 7.05 (s, 1 H), 7.16 (s, 1 H), 7.19–7.21 (m, 1 H), 7.26–7.29 (m, 1 H), 7.34–7.40 (m, 5 H), 7.45–7.47 (m, 2 H), 7.51–7.55 (m, 3 H). ^{13}C NMR (CD_3OD): δ = 18.35, 26.11, 41.19, 50.60, 72.15, 103.21, 112.82, 112.86, 113.32, 121.40, 124.40, 128.63, 128.69, 128.84, 128.96, 129.33, 129.85, 130.81, 133.53, 136.15, 139.27, 142.17, 153.92, 168.14, 174.84. ESI-MS (*m*/*z*): 468 $[M + H]^+$. Anal. ($C_{29}H_{29}N_3O_3 \cdot 0.3H_2O$): C, H, N.

***N*-(3-Phenylpropenoyl)-glycine-5-benzyloxytryptamide (26)** was prepared from **17a** (0.27 g, 1.32 mmol) and 5-benzyloxytryptamine (0.40 g, 1.32 mmol) using pivaloyl chloride (0.16 mL, 1.32 mmol) and two portions of Et_3N (0.40 mL, 2.89 mmol) according to the syntheses of **13b**. The mixture was stirred at rt for 3 h (Sam *et al.* 1959). The mixture was washed twice with 30% citric acid, ones with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified by flash chromatography using a manual gradient of petrol ether:ethyl

acetate (60:40→0:100) (17%). ^{1}H NMR (CD_3OD): δ = 2.92 (t, ^{3}J = 7.3 Hz, 2 H), 3.50 (t, ^{3}J = 7.3 Hz, 2 H), 3.94 (s, 2 H), 5.09 (s, 2 H), 6.62–6.65 (m, 1 H), 6.80–6.83 (m, 1 H), 7.05 (s, 1 H), 7.16–7.16 (m, 1 H), 7.20–7.22 (m, 1 H), 7.26–7.29 (m, 1 H), 7.34–7.41 (m, 5 H), 7.45–7.47 (m, 2 H), 7.53–7.56 (m, 3 H). ^{13}C NMR (CD_3OD): δ = 26.09, 41.23, 43.92, 72.43, 103.55, 112.89, 112.89, 113.44, 121.37, 124.42, 128.61, 128.65, 128.83, 129.01, 129.32, 129.82, 130.78, 133.63, 136.16, 139.28, 142.30, 154.01, 168.86, 171.29. ESI-MS (*m*/*z*): 454 $[M + H]^+$. Anal. ($C_{28}H_{27}N_3O_3$): C, H, N.

***N*-(3-Phenylpropanoyl)-L-alanine (27a).** 3-Phenyl propanoic acid (0.75 g, 5.00 mmol) was dissolved in thionyl chloride (1.1 mL). The mixture was refluxed gently for 1.5 h, and the solvents were evaporated. *N*-(3-Phenylpropanoyl)-L-alanine was prepared from 3-phenyl propanoic acid chloride in diethyl ether (30 mL) and L-alanine (0.45 g, 5.00 mmol) in 1 M NaOH (40 mL) according to the syntheses of **12b**. The H_2O phase was made acidic with 2 M HCl, and the product was extracted with DCM. The organic phase was dried and evaporated. The product was used without further purification.

***N*-(3-Phenylpropanoyl)-L-alanine *N*-hydroxysuccinimide ester (27b)** was prepared from **27a** (0.80 g, 3.62 mmol) and *N*-hydroxysuccinimide (0.42 g, 3.62 mmol) using *N*,*N*'-Dicyclohexylcarbodiimide (0.75 g, 3.62 mol) in dry ACN according to the syntheses of **12c**. The reaction was stirred at -20°C for 2 h. The yield was 60%. The product was used without further purification.

***N*-(3-Phenylpropanoyl)-L-alanine-5-benzyloxytryptamide (27).** Compound **27b** (0.45 g, 1.40 mmol) was dissolved in THF and 5-benzyloxytryptamine (0.42 g, 1.40 mmol) was added. 5-Benzyloxytryptamine did not dissolve completely. The mixture was stirred at rt overnight. More THF was added. Two portions of Et_3N (0.20 mL, 1.40 mmol) was yet added to complete the reaction. The mixture was stirred overnight. THF was evaporated and the residue was dissolved in DCM and washed twice with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was dried and evaporated. The product was purified by flash chromatography using a manual gradient of DCM:MeOH (99:1→98:2) (36%). ^{1}H NMR (CD_3OD): δ = 1.20–1.22 (m, 3 H), 2.47–2.50 (m, 2 H), 2.85–2.88 (m, 4 H), 3.41–3.44 (m, 2 H), 4.24–4.28 (m, 1 H), 5.09 (s, 2 H), 6.82–6.84 (m, 1 H), 7.03 (s, 1 H), 7.12–7.18 (m, 4 H), 7.21–7.24 (m, 3 H), 7.27–7.30 (m, 1 H), 7.34–7.37 (m, 2 H), 7.46–7.47 (m, 2 H). ^{13}C NMR

(CD_3OD): δ = 18.08, 26.01, 32.53, 38.43, 41.00, 50.22, 72.11, 103.21, 112.69, 112.86, 113.24, 124.34, 127.08, 128.58, 128.62, 128.86, 129.24, 129.28, 129.31, 133.45, 139.13, 141.88, 153.85, 174.68, 174.74. ESI-MS (*m*/*z*): 470 $[M + H]^+$. Anal. ($C_{29}H_{31}N_3O_3 \cdot 0.3H_2O$): C, H, N.

***N*-3-(4-Acetoxyphenylpropenoyl)-tryptamide (28a). 21c** (0.51 g, 2.49 mmol) was dissolved in DCM and thionyl chloride (5 mL) was added. The mixture was refluxed gently for 1 h, and the solvents were evaporated. Tryptamine (0.40 g, 2.49 mmol) and Et_3N (0.35 mL, 2.49 mmol) were dissolved in DCM and 3-(4-acetoxyphenyl)-propenoic acid chloride was added through the dropping funnel to the mixture. The mixture was stirred at rt overnight. The mixture was washed three times with 30% citric acid, once with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The solvent was dried and evaporated. The product was washed with DCM (50%). ^1H NMR (CD_3OD): δ = 2.27 (s, 3 H), 3.02 (t, ^3J = 7.3 Hz, 2 H), 3.61 (t, ^3J = 7.3 Hz, 2 H), 6.54 (d, ^3J = 15.8 Hz, 1 H), 6.98–7.02 (m, 1 H), 7.07–7.13 (m, 4 H), 7.32–7.34 (m, 1 H), 7.51 (d, ^3J = 15.8 Hz, 1 H), 7.55–7.59 (m, 3 H).

***N*-3-(4-Hydroxyphenylpropenoyl)-tryptamide (28). 28a** (0.43 g, 1.23 mmol) was dissolved in MeOH. The flask was under argon at 0°C. 1 M CH_3ONa (2.47 mL, 2.47 mmol) in MeOH was added. The reaction was over in 15 min (Boiadjiev and Lightner 1996). MeOH was acidified with 3 M HCl. The product started to precipitate when DCM was added. The product was filtered (84%). ^1H NMR (CD_3OD): δ = 3.00 (t, ^3J = 7.3 Hz, 2 H), 3.59 (t, ^3J = 7.3 Hz, 2 H), 6.39 (d, ^3J = 15.7 Hz, 1 H), 6.78–6.79 (m, 2 H), 6.98–7.01 (m, 1 H), 7.06–7.09 (m, 2 H), 7.32–7.33 (m, 1 H), 7.38–7.40 (m, 2 H), 7.45 (d, ^3J = 15.7 Hz, 1 H), 7.57–7.59 (m, 1 H). ^{13}C NMR (CD_3OD): δ = 26.42, 41.63, 112.21, 113.32, 116.71, 118.60, 119.31, 119.59, 122.32, 123.41, 127.78, 128.80, 130.54, 138.19, 141.70, 160.49, 169.30. ESI-MS (*m*/*z*): 307 $[M + H]^+$. Anal. ($C_{19}H_{18}N_2O_2$): C, H, N.

***N*-3-Phenylpropenoyl-tryptamide (29)** was prepared from tryptamine (0.20 g, 1.25 mmol) in DCM, 1 M NaOH (8 mL, 8 mmol) and 3-phenyl propenoic acid chloride in DCM according to the syntheses of **12b**. The mixture was stirred strongly for 1.5 h. The mixture was washed three times with 30% citric acid, ones with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was dried and evaporated (64%). ^1H NMR

(CD_3OD): δ = 3.02 (t, ^3J = 7.3 Hz, 2 H), 3.61 (t, ^3J = 7.3 Hz, 2 H), 6.56–6.59 (m, 1 H), 6.98–7.01 (m, 1 H), 7.07–7.10 (m, 2 H), 7.32–7.39 (m, 4 H), 7.51–7.54 (m, 3 H), 7.58–7.59 (m, 1 H). ^{13}C NMR (DMSO-d_6): δ = 25.19, 39.59, 111.30, 111.76, 118.16, 118.20, 120.85, 122.40, 122.57, 127.19, 127.39, 128.81, 129.24, 134.94, 136.23, 138.38, 164.87. ESI-MS (*m*/*z*): 291 $[M + H]^+$. Anal. ($C_{19}H_{18}N_2O \cdot 0.02H_2O$): C, H, N.

***N*-3-Phenylpropanoyl-tryptamide (30).** 3-Phenyl propanoic acid (0.40 g, 2.7 mmol) was dissolved in thionyl chloride (0.58 mL, 8.0 mmol). The mixture was refluxed gently for 1.5 h. Thionyl chloride was evaporated. *N*-3-Phenylpropanoyl-tryptamide was prepared from tryptamine (0.36 g, 2.2 mmol) in DCM, 1 M NaOH (13 mL, 13 mmol) and 3-phenyl propanoic acid chloride in DCM according to the syntheses of **12b**. The mixture was stirred strongly for 1 h. The mixture was washed three times with 30% citric acid, ones with sat. NaCl aq. and twice with sat. $NaHCO_3$ aq. The organic phase was dried and evaporated (76%). ^{1}H NMR (CD_3OD): δ = 2.42–2.45 (m, 2 H), 2.85–2.88 (m, 4 H), 3.42–3.44 (m, 2 H), 6.95 (s, 1 H), 6.98–7.01 (m, 1 H), 7.06–7.09 (m, 1 H), 7.14–7.18 (m, 3 H), 7.22–7.25 (m, 2 H), 7.31–7.33 (m, 1 H), 7.52–7.54 (m, 1 H). ^{13}C NMR (DMSO-d_6): δ = 25.14, 31.06, 37.05, 39.40, 111.25, 111.79, 118.10, 118.13, 120.78, 122.47, 125.73, 127.16, 128.11, 128.14, 136.17, 141.33, 171.10. ESI-MS (*m*/*z*): 293 $[M + H]^+$. Anal. ($C_{19}H_{20}N_2O$): C, H, N.

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8 N^{ε}-THIOACETYL-LYSINE-CONTAINING TRI-, TETRA-, AND PENTAPEPTIDES AS SIRT1 AND SIRT2 INHIBITORS *

Abstract: A series of N^{ε}-thioacetyl-lysine containing tri-, tetra-, and pentapeptides were studied as SIRT1 and SIRT2 inhibitors. The peptides were based on the α-tubulin and p53 protein sequences. The most potent peptides had nanomolar inhibitory activities against SIRT1 and micromolar inhibitory activities against SIRT2. Lys-Lys(ε-thioAc)-Leu-Met and Lys-Lys(ε-thioAc)-Leu are highly potent and selective SIRT1 inhibitors.

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8.1 Introduction

A new series of short N^{ε}-thioacetyl-lysine containing tri-, tetra-, and pentapeptides was designed based on two substrate sequences; human α-tubulin (38–42) and human p53 tumor suppressor protein (380–384) (figure 8.1), in order to determine important amino acid side chains and minimum peptide length for SIRT1 and SIRT2 inhibitory activity. Fatkins and Zheng have reported earlier pentapeptide **6** with an IC_{50} value of about 10 μM (the exact value was not reported) (2008). Garske and Denu have shown that for the peptide substrates the amino acids beyond positions –2 and +2 (the second residues towards the N-terminus and C-terminus, respectively, calculating from the N^{ε}-acetyl-lysine) are not necessary for efficient binding and activity of sirtuins (Garske and Denu 2006). In this study we show that several pentapeptides are potent SIRT1 and SIRT2 inhibitors. Further truncation by removal of the –2 and +2 amino acids is tolerated by SIRT1 but not by SIRT2, which provides access to potent and selective substrate-based SIRT1 inhibitors.

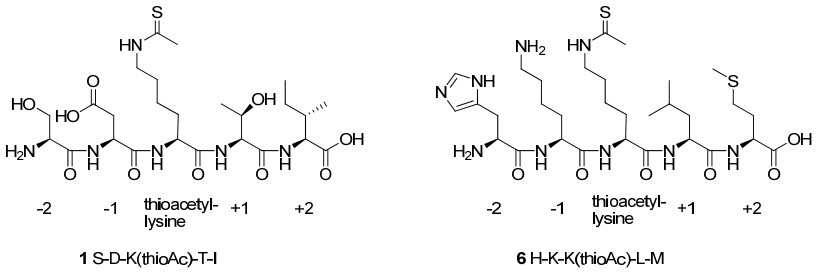


Figure 8.1. Human α-tubulin (S-D-K(thioAc)-T-I) **1** and human p53 tumor suppressor protein (H-K-K(thioAc)-L-M) **6** sequences.

8.2 Synthetic chemistry

Peptides were synthesized manually or on a peptide synthesizer using an Fmoc strategy with TBTU or HBTU and DIPEA as the coupling reagents and Wang resin or NovaSyn TGA as the solid phase. N^{α}-Fmoc-N^{ε}-thioacetyl-lysine was synthesized as described in the literature (Fatkins *et al.* 2006). All other amino acids used were common natural amino acids. Reference compound **24** (EX-527) was synthesized as described in the

literature and tested as a racemate (Napper *et al.* 2005). The inhibitory activities were tested in a Fluor de Lys fluorescence-based assay.

8.3 Results and discussion

The synthesized peptides and their inhibitory activities are presented in table 8.1. Peptides **1**–**5** based on human α-tubulin (38–42) sequence and peptides **6**–**9** based on human p53 protein (380–384) sequence were synthesized to study the effect of the peptide length. Pentapeptide **6** has been reported earlier by Fatkins and Zheng with an IC_{50} value of about 10 μM (the exact value was not reported) (2008). In general, the p53–based peptides gave better inhibitory activities for SIRT1 and SIRT2 than the α-tubulin–based peptides. This observation prompted us to synthesize peptides **10**–**15**, in which one or two amino acids of the α-tubulin sequence were replaced by the corresponding amino acids from the p53 sequence. Peptides **16**–**23** with the alanine replacements on the α-tubulin and p53 sequences were synthesized to study the importance of the individual amino acid side chains.

At the –2 position, the serine residue of the α-tubulin–based sequence **1** was replaced by either a histidine or an alanine residue, resulting in peptides **10** and **16**, respectively. Serine and histidine side chains are able to form hydrogen bonds while an alanine side chain is not. All three peptides were equipotent against SIRT1 and SIRT2 indicating that the hydrogen bonding of the –2 side chain is not prerequisite for a good inhibitory activity.

Removal of the –2 amino acid resulted in the α-tubulin–based tetrapeptide **3**, which, interestingly, was equipotent against SIRT1 but almost three times less potent against SIRT2 than pentapeptide **1**. The same effect was observed with the p53–based sequences; tetrapeptide **8** was equipotent against SIRT1 but almost eight times less potent against SIRT2 than pentapeptide **6**. It seems that SIRT1 and SIRT2 differ in their binding interactions with the –2 amino acid of the peptides. For SIRT2, the presence of an amino acid at this position was more important than the identity of its side chain, indicating that the main chain interactions of the –2 residue may be more relevant than the side chain interactions.

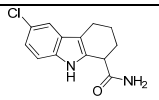
At the –1 position, the aspartic acid residue of the α-tubulin–based peptide **1** was replaced either by a lysine or an alanine residue, resulting in peptides **11** and **17**, respectively. Both peptides were clearly more potent than the parent peptide **1** for SIRT1 and SIRT2. In fact, peptides **11** and **17** were slightly more potent for SIRT1 and equipotent for SIRT2 as compared to the most potent p53–based peptide **6**. It is clear that a negatively charged aspartic acid residue at the position –1 was the reason for the lower inhibitory activities of the α-tubulin–based sequences **1–4** compared to the p53–based sequences **6–9**. However, it is noteworthy that the sequence **1** is based on a SIRT2 substrate, and the studied replacements of the aspartic acid residue increased the SIRT2 inhibitory activity only five–fold but SIRT1 inhibitory activity about 25–fold.

At the +1 position, the threonine residue of the α-tubulin–based peptide **1** was replaced by either an alanine or a leucine residue, resulting in peptides **18** and **12**, respectively. For SIRT1, the replacement by alanine gave an equipotent peptide but the replacement by leucine resulted in a clearly less potent peptide. For SIRT2, both replacements resulted in less potent peptides. In the p53–based sequences, peptide **6** with a leucine residue and peptide **22** with an alanine residue at the position +1 are equipotent.

At the +2 position, the isoleucine residue of the α-tubulin–based peptide **1** was replaced by a methionine or an alanine residue, resulting in peptides **13** and **19**, respectively. The replacement by a methionine residue slightly increased and the replacement by an alanine residue slightly decreased the inhibitory activity. The same trend was observed with the p53–based sequences. The +2 methionine residue increased the inhibitory activity compared to isoleucine (**15** vs. **12**) or alanine (**6** vs. **23**).

Removal of the amino acid at the position +2 in the α-tubulin–based peptide **1** resulted in peptide **2**, which had slightly decreased inhibitory activities for SIRT1 and SIRT2. The effect of the removal of the amino acid at position +2 was also confirmed with the p53–based sequences **6** and **7**. The inhibitory activity is not significantly dependent on the presence of an amino acid at the position +2.

Table 8.1. Peptide sequences and their inhibitory activities for SIRT1 and SIRT2.

Peptide	Sequence [a]	IC_{50} (μM) for SIRT1[b]		IC_{50} (μM) for SIRT2[b]	
Peptides based on the human α-tubulin protein sequence (38-42)					
1	Ser-Asp-Lys(ε-thioAc)-Thr-Ile	5.0	(4.3-5.9)	29.1	(18.7-45.3)
2	Ser-Asp-Lys(ε-thioAc)-Thr	11.1	(9.5-12.9)	44.0	(27.5-70.3)
3	Asp-Lys(ε-thioAc)-Thr-Ile	4.5	(3.8-5.4)	83.4	(44.9-154.8)
4	Asp-Lys(ε-thioAc)-Thr	12.0	(9.7-14.9)	175	(109-283)
5	Ser-Asp-Lys(ε-thioAc)	11.8 ± 1.9% at 200 μM[c]		13.9 ± 4.4% at 200 μM[c]	
Peptides based on the human p53 protein sequence (380–384)					
6	His-Lys-Lys(ε-thioAc)-Leu-Met	0.33	(0.27-0.40)	6.4	(5.3-7.7)
7	His-Lys-Lys(ε-thioAc)-Leu	0.48	(0.32-0.71)	19.9	(17.4-22.6)
8	Lys-Lys(ε-thioAc)-Leu-Met	0.33	(0.26-0.42)	49.8	(37.9-65.6)
9	Lys-Lys(ε-thioAc)-Leu	0.57	(0.38-0.84)	151	(104-218)
p53 Replacements on the human α-tubulin sequence (38–42)					
10	**His**-Asp-Lys(ε-thioAc)-Thr-Ile	4.3	(2.9-6.3)	31.1	(23.8-40.8)
11	Ser-**Lys**-Lys(ε-thioAc)-Thr-Ile	0.18	(0.15-0.21)	6.9	(6.2-7.7)
12	Ser-Asp-Lys(ε-thioAc)-**Leu**-Ile	23.2	(20.8-25.8)	238	(186-305)
13	Ser-Asp-Lys(ε-thioAc)-Thr-**Met**	1.4	(1.0-1.9)	10.6	(0.9-12.8)
14	**His-Lys**-Lys(ε-thioAc)-Thr-Ile	0.23	(0.19-0.27)	7.6	(6.7-8.6)
15	Ser-Asp-Lys(ε-thioAc)-**Leu-Met**	10.8	(8.2-14.2)	92.9	(80.0-107.8)
Alanine replacements on the α-tubulin protein sequence (38–42)					
16	**Ala**-Asp-Lys(ε-thioAc)-Thr-Ile	3.2	(2.4-4.3)	37.4	(17.3-80.9)
17	Ser-**Ala**-Lys(ε-thioAc)-Thr-Ile	0.22	(0.18-0.26)	5.5	(3.1-10.1)
18	Ser-Asp-Lys(ε-thioAc)-**Ala**-Ile	6.7	(4.5-9.8)	72.8	(62.3-85.1)
19	Ser-Asp-Lys(ε-thioAc)-Thr-**Ala**	8.8	(5.0-15.6)	84.6	(31.8-224.9)
Alanine replacements on the p53 protein sequence (380–384)					
20	**Ala**-Lys-Lys(ε-thioAc)-Leu-Met	0.38	(0.30-0.49)	8.5	(7.1-10.3)
21	His-**Ala**-Lys(ε-thioAc)-Leu-Met	0.55	(0.38-0.79)	16.5	(8.2-33.0)
22	His-Lys-Lys(ε-thioAc)-**Ala**-Met	0.18	(0.14-0.23)	3.8	(1.9-7.5)
23	His-Lys-Lys(ε-thioAc)-Leu-**Ala**	0.90	(0.69-1.17)	33.3	(23.5-47.2)
Reference compound EX-527					
24 [d]		0.28	(0.23-0.34)	14.0	(8.0-25.0)

[a] The sequences were tested with free amino- and carboxy-terminals. [b] 95% Confidence intervals for IC_{50} values are given in parentheses. Each experiment was repeated at least three times. [c] Inhibition-% at 200 μM ± standard deviation. The IC_{50} value could not be determined due to the weak inhibitory activity. [d] Compound **24** was tested as a racemate.

N^{α}-Fmoc-N^{ε}-thioacetyl-lysine and N^{α}-acetyl-N^{ε}-thioacetyl-lysine have been reported to show no inhibitory activity towards SIRT1 (Fatkins *et al.* 2006) but the inhibitory

activities of tri- or tetrapeptides against SIRT1 and SIRT2 have not been reported before. Removal of the amino acids from the –2 and +2 positions did not affect the inhibitory activity for SIRT1 but significantly decreased the inhibitory activity for SIRT2. The α-tubulin–based tripeptide **4** and the p53–based tripeptide **9** had IC_{50} values of 12.0 μM and 0.57 μM for SIRT1 and 175 μM and 151 μM for SIRT2, respectively. Removal of the amino acids from the positions +1 and +2 resulted in substantial loss of activity against both enzymes. The α-tubulin–based tripeptide **5** had a significantly decreased inhibitory activity: 11.8% inhibition at 200 μM against SIRT1 and 13.9% inhibition at 200 μM against SIRT2.

Two of the p53–based tri- and tetrapeptides showed increased selectivity for SIRT1 over SIRT2; tetrapeptide **8** and, in particular, tripeptide **9** were more selective for SIRT1 than the well-known SIRT1 selective inhibitor **24**, which was used as the reference compound. None of the sequences showed selectivity for SIRT2.

8.4 Conclusions

In conclusion, p53– and α-tubulin–based N^{ε}-thioacetyl-lysine containing tri-, tetra-, and pentapeptides are potent SIRT1 and SIRT2 inhibitors. The p53–based sequences gave overall better inhibitory activities than the α-tubulin–based sequences, mainly due to the unfavorable aspartic acid residue in the position –1 of the α-tubulin sequence. The studied series shows that the correct selection of side chains is important for good inhibitory activity. In fact, the difference in the IC_{50} values between the most potent and the least potent pentapeptide inhibitors is 130-fold for SIRT1 and 46-fold for SIRT2. Four of the most potent SIRT1 inhibitors **11**, **14**, **17**, and **22** had IC_{50} values of 180-230 nM, equipotent to the reference compound **24**. The most potent SIRT2 inhibitor **22** had an IC_{50} value of 3.8 μM. These peptides are among the most potent SIRT1 and SIRT2 inhibitors published so far. In addition, the peptides **8** and **9** were selective for SIRT1 over SIRT2, indicating that there are differences in the main chain interactions. As it has been claimed that it is difficult to design peptidomimetics for peptides larger than four amino acids (Adessi and Soto 2002), peptides **8** and **9** provide a promising starting point for the development of small peptidomimetic SIRT1 inhibitors.

8.5 Synthetic procedures and analytical data

General. Fmoc-amino acids in the peptide synthesis were commercial (Fluka or GL Biochem) except for N^{α}-Fmoc-N^{ε}-thioacetyl-lysine, and had the following side chain protection: O-*t*-Bu for Asp, Trt for His, Boc for Lys, and *t*-Bu for Thr and Ser.

***N*$^{\alpha}$-Fmoc-*N*$^{\varepsilon}$-thioacetyl-lysine.** N^{α}-Fmoc-N^{ε}-thioacetyl-lysine was synthesized as described previously by Fatkins *et al.*(2006). Fmoc-Lys-OH · HCl (2.2 g, 5.43 mmol) was suspended in ethanol (11 mL) and made alkaline with 10% (w/v) aqueous solution of Na_2CO_3 (10 mL) at 0^{o}C. Ethyl dithioacetate (0.69 mL, 5.98 mmol) was added at rt and the mixture was stirred overnight. The mixture was diluted with water (5 mL) and ethanol was removed under reduced pressure. The residual aqueous solution was acidified with 6 M HCl to pH ~ 1–2 and extracted three times with DCM. The combined organic phases were washed three times with sat. NaCl aq., dried over anhydrous Na_2SO_4, filtered and evaporated to yield white solid (1.98 g, 85%) which was used directly for peptide syntheses. ^{1}H NMR (DMSO-d_6): δ = 1.33-1.42 (m, 2 H), 1.51-1.77 (m, 4 H), 2.38 (s, 3 H), 3.43-3.49 (m, 2 H), 3.92-3.96 (m, 1 H), 4.21-4.30 (m, 3 H), 7.32-7.35 (m, 2 H), 7.40-7.43 (m, 2 H), 7.62-7.64 (m, NH), 7.72-7.73 (m, 2 H), 7.88-7.89 (m, 2 H), 9.95 (s, NH), 12.56 (s, OH).

***N*$^{\alpha}$-Fmoc-*N*$^{\varepsilon}$-thioacetyl-lysine attachment to Wang resin.** N^{α}-Fmoc-N^{ε}-thioacetyl-lysine attachment to Wang resin was done as described by Novabiochem (Novabiochem). Wang resin 350 mg (0.35 mmol, concentration about 1 mmol/g) was placed to the flask and sufficient amount of DMF was added to cover the resin. Resin was allowed to swell for 30 min. N^{α}-Fmoc-N^{ε}-thioacetyl-lysine (1.18 g, 2.77 mmol) was dissolved in dry DCM (7 mL). A couple of drops to DMF was added to complete dissolution. The flask was placed on ice. Diisopropylcarbodiimide (0.22 g, 1.8 mmol) was added. The flask was closed with a calcium chloride drying tube and the mixture was stirred for 20 min at 0^{o}C. DCM was evaporated, the residue was dissolved in small amount of DMF (5 mL) and added to the Wang resin solution. 4-Dimethylaminopyridine (0.004 g, 0.04 mmol) was yet added to the amino acid / resin solution and the flask was closed. The mixture was stood at rt for 1.5 h with occasional swirling. The mixture was placed to the syringe and washed five times with DMF and five times with DCM. Loaded resin was used directly for the synthesis of peptide **5**.

Manual peptide synthesis. Peptides **1**–**15**, **18**, **20**, and **23** were synthesized manually using Fmoc strategy with TBTU/DIPEA as the coupling reagent and Wang resin as solid phase (Fluka). The first resin-bound N^{α}-Fmoc-amino acid (300-350 mg) was swelled for 1 h in 3 mL of DMF. The N-terminal amino acid was deprotected with 5 mL of 20% (v/v) piperidine in DMF for 10-15 min and washed 5 times with DMF. The N^{α}-Fmoc-amio acids (Fluka) were dissolved in 3-5 mL of DMF and preactivated by mixing with TBTU (4 eq)/ DIPEA (10eq). The amino acid was added on the resin and shaken at rt for 45-50 min and washed five times with DMF. Cycles of deprotection of Fmoc and coupling with the subsequent amino acids were repeated until the desired peptide-bond resin was completed. The resin was washed ones with acetic acid, five times with DCM and twice with methanol and evaporated under vacuum overnight. The product was cleaved from the resin with 5 mL of Reagent K (cleavage cocktail which contains 82.5% TFA, 5% H_2O, 5% phenol, 5% thioanisol, and 2.5% ethanedithiol; 5mL per 300-350 mg per resin) and reacted at rt for 45-90 min. The crude product was precipitated with cold ether, collected by centrifugation (5 min at 4000 rpm) and washed ones with cold ether to remove any residual scavengers. The crude product was dried in vacuum overnight.

Peptide synthesizer. The peptides **16**, **17**, **19**, **21**, and **22** were synthesized on an Apex 396 DC multiple peptide synthesizer (Advanced ChemTech, Louisville, KY) using Fmoc strategy with HBTU/DIPEA as the coupling reagent and Wang resin and NovaSyn TGA as the solid phase (Fluka, Novabiochem and GL Biochem (Shanghai)). The cleavage was done using the mixture of 95% TFA, 3% ethanedithiol, 1% tris-isopropylsilane, and 1% H_2O.

HPLC purification. Peptides were purified by preparative HPLC (Shimadzu LC-10A*vp* (Fennolab, Fenno Medical Oy)) described in Chapter 4.2.

NMR Data. ^{1}H NMR shifts are assigned in tables 8.2-8.24. Overlapping solvent residual signals were observed for acetonitrile $\underline{C}H_3$ and acetate $\underline{C}H_3$. ^{13}C NMR shifts are also assigned below. Solvent residual signals were observed in the ^{13}C- and APT-spectra as follows: acetonitrile $\underline{C}H_3$ at 2.2-2.4 ppm in D_2O and at 1.4 ppm in DMSO-*d6*; acetate $\underline{C}H_3$ at 22.0-22.5 ppm in D_2O and at 21.3-21.4 in DMSO-*d6*; trifuoroacetate $\underline{C}F_3$ at 117.8 ppm and $\underline{C}$=O at 164.3 ppm in D_2O.

Table 8.2. Chemical shifts of proton resonance for peptide **1** (Chemical shifts in ppm (D_2O)).

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Ser	**4.17** (t, ^3J= 4.9 Hz, CH)	**4.01** (d, ^3J= 4.9 Hz, CH_2)	-	-	-	-
Asp	**4.78-4.84** (m, CH)	**2.77** (dd, ^3J= 16.5 Hz, ^2J= 8.0 Hz, CH) **2.89** (dd, ^3J= 16.4 Hz, ^2J= 5.6 Hz, CH)	-	-	-	-
Lys (thioAc)	**4.40** (dd, ^3J= 8.6 Hz, ^3J= 5.7 Hz, CH)	**1.74-1.83** (m, CH) **1.85-1.94** (m, CH)	**1.35-1.53** (m, CH_2)	**1.65-1.74** (m, CH_2)	**3.61** (t, ^3J= 7.0 Hz, CH_2)	**2.53** (s, CH_3)
Thr	**4.36** (d, ^3J= 5.2 Hz, CH)	**4.18-4.23** (m, CH)	**1.23** (d, ^3J= 6.4 Hz, CH)	-	-	-
Ile	**4.25** (d, ^3J= 5.9 Hz, CH)	**1.89-1.98** (m, CH)	**0.96** (d, ^3J= 6.9 Hz, CH_3) **1.19-1.29** (m, CH) **1.44-1.54** (m, CH)	**0.93** (t, ^3J= 7.5 Hz, CH_3)	-	-

Table 8.3. Chemical shifts of proton resonance for peptide **2** (Chemical shifts in ppm (DMSO-d_6)).

Residue	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Ser	-	**3.71-3.80** (m, CH)	**3.62-3.68** (m, CH) **3.70-3.76** (m, CH)	-	-	-	-
Asp	**8.68** (s, NH)	**2.54** (dd, ^3J= 14.8 Hz, ^2J= 7.9 Hz, CH) **2.69** (dd, ^3J= 16.3 Hz, ^2J= 5.4 Hz, CH)	**4.62** (s, CH)	-	-	-	-
Lys (thioAc)	**8.06** (d, ^3J= 8.1 Hz, NH)	**4.27-4.32** (m, CH)	**1.52-1.61** (m, CH) **1.69-1.79** (m, CH)	**1.26-1.38** (m, CH_2)	**1.47-1.61** (m, CH_2)	**3.39-3.47** (m, CH_2)	**9.98** (s, NH) **2.38** (s, CH_3)
Thr	**7.77** (d, ^3J= 7.7 Hz, NH)	**4.04-4.09** (m, CH)	**4.04-4.09** (m, CH)	**1.03** (d, ^3J= 5.9 Hz, CH_3)	-	-	-

Table 8.4. Chemical shifts of proton resonance for peptide **3** (Chemical shifts in ppm (DMSO-d_6)).

Residue	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Asp	-	**3.85-3.91** (m, CH)	**2.44** (dd, ^3J= 16.6 Hz, ^2J= 8.8 Hz, CH) **2.61** (dd, ^3J= 16.6 Hz, ^2J= 4.4 Hz, CH)	-	-	-	-
Lys (thioAc)	**8.54** (d, ^3J= 7.1 Hz, NH)	**4.31-4.37** (m, CH)	**1.52-1.62** (m, CH) **1.70-1.82** (m, CH)	**1.28-1.45** (m, CH_2)	**1.49-1.62** (m, CH_2)	**3.39-3.48** (m, CH_2)	**10.05** (s,NH) **2.38** (s, CH_3)
Thr	**8.09** (d, ^3J= 8.3 Hz, NH)	**4.17-4.21** (m, CH)	**3.94-4.01** (m, CH)	**1.05** (d, ^3J= 6.3 Hz, CH_3)	-	-	-
Ile	**7.68** (d, ^3J= 8.3 Hz, NH)	**4.11-4.15** (m, CH)	**1.73-1.82** (m, CH)	**0.80-0.88** (m, CH_3) **1.11-1.21** (m, CH) **1.36-1.48** (m, CH)	**0.80-0.88** (m, CH_3)	-	-

Table 8.5. Chemical shifts of proton resonance for peptide **4** (Chemical shifts in ppm (DMSO-d_6)).

Residue	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Asp	-	**3.98** (dd, ^3J= 8.6 Hz, ^2J= 4.2 Hz, CH)	**2.54** (dd, ^3J= 15.2 Hz, ^2J= 8.8 Hz, CH) **2.69** (dd, ^3J= 17.0 Hz, ^2J= 4.2 Hz, CH)	-	-	-	-
Lys (thioAc)	**8.53** (d, ^3J= 7.9 Hz, NH)	**4.38-4.43** (m, CH)	**1.48-1.61** (m, CH) **1.70-1.81** (m, CH)	**1.27-1.43** (m, CH_2)	**1.48-1.61** (m, CH_2)	**3.39-3.49** (m, CH_2)	**9.97** (s, NH) **2.38** (s, CH_3)
Thr	**7.96** (d, ^3J= 8.1 Hz, NH)	**4.10-4.15** (m, CH)	**4.10-4.15** (m, CH)	**1.06** (d, ^3J= 6.2 Hz, CH_3)	-	-	-

Table 8.6. Chemical shifts of proton resonance for peptide **5** (Chemical shifts in ppm (D_2O)).

Residue	**$C^{\alpha}H$**	**$C^{\beta}H$**	**$C^{\gamma}H$**	**$C^{\delta}H$**	**$C^{\varepsilon}H$**	**Others**
Ser	**4.19-4.21** (m, CH)	**3.99-4.06** (m, CH_2)	-	-	-	-
Asp	**4.73-4.79** (m, CH)	**2.81** (dd, ^3J= 16.8 Hz, ^2J= 8.6 Hz, CH) **2.94** (dd, ^3J= 16.8 Hz, ^2J= 4.9 Hz, CH)	-	-	-	-
Lys (thioAc)	**4.27-4.30** (m, CH)	**1.76-1.82** (m, CH) **1.85-1.94** (m, CH)	**1.37-1.47** (m, CH_2)	**1.64-1.76** (m, CH_2)	**3.62** (t, ^3J = 7.0 Hz, CH_2)	**2.52** (s, CH_3)

Table 8.7. Chemical shifts of proton resonance for peptide **6** (Chemical shifts in ppm (D_2O)).

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
His	**4.30-4.43** (m, CH)	**3.39-3.48** (m, CH_2)	-	-	-	**7.45** (s, 4'CH) **8.72** (s, 2´CH) (imidazole)
Lys	**4.30-4.43** (m, CH)	**1.75-1.91** (m, CH_2)	**1.36-1.53** (m, CH_2)	**1.63-1.79** (m, CH_2)	**3.02** (t, ^3J = 7.6 Hz, CH_2)	-
Lys (thioAc)	**4.30-4.43** (m, CH)	**1.75-1.91** (m, CH_2)	**1.36-1.53** (m, CH_2)	**1.63-1.79** (m, CH_2)	**3.63** (t, ^3J = 7.0 Hz, CH_2)	**2.52** (s, CH_3)
Leu	**4.30-4.43** (m, CH)	**1.61-1.76** (m, CH_2)	**1.61-1.76** (m, CH)	**0.91** (d, ^3J = 5.9 Hz, CH_3) **0.96** (d, ^3J = 5.7 Hz, CH_3)	-	-
Met	**4.30-4.43** (m, CH)	**1.94-2.05** (m, CH) **2.14-2.20** (m, CH)	**2.45-2.61** (m, CH_2)	**2.12** (s, CH_3)	-	-

Table 8.8. Chemical shifts of proton resonance for peptide **7** (Chemical shifts in ppm (D_2O)).

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
His	**4.29-4.47** (m, CH)	**3.38-3.49** (m, CH_2)	-	-	-	**7.46** (s, 4'CH) **8.75** (s, 2´CH) (imidazole)
Lys	**4.29-4.47** (m, CH)	**1.78-1.91** (m, CH_2)	**1.41-1.53** (m, CH_2)	**1.66-1.78** (m, CH_2)	**3.01** (t, ^3J= 7.3 Hz, CH_2)	-
Lys (thioAc)	**4.29-4.47** (m, CH)	**1.78-1.91** (m, CH_2)	**1.41-1.53** (m, CH_2)	**1.66-1.78** (m, CH_2)	**3.63** (t, ^3J= 6.8 Hz, CH_2)	**2.52** (s, CH_3)
Leu	**4.29-4.47** (m, CH)	**1.65-1.75** (m, CH_2)	**1.65-1.75** (m, CH)	**0.92** (d, ^3J= 5.7 Hz, CH_3) **0.98** (d, ^3J= 5.9 Hz, CH_3)	-	-

Table 8.9. Chemical shifts of proton resonance for peptide **8** (Chemical shifts in ppm (D_2O)).

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Lys	**4.04** (t, ^3J= 6.5 Hz, CH)	**1.61-1.87** (m, CH) **1.89-1.97** (m, CH)	**1.37-1.54** (m, CH_2)	**1.61-1.87** (m, CH_2)	**3.03** (t, ^3J= 7.6 Hz, CH_2)	-
Lys (thioAc)	**4.36** (t, ^3J= 7.2 Hz, CH)	1.**61-1.87** (m, CH) **1.89-1.97** (m, CH)	**1.37-1.54** (m, CH_2)	**1.61-1.87** (m, CH_2)	**3.62** (t, ^3J= 7.0 Hz, CH_2)	**2.53** (s, CH3)
Leu	**4.37-4.43** (m, CH)	**1.63-1.74** (m, CH_2)	**1.63-1.74** (m, CH)	**0.92** (d, ^3J = 5.9 Hz, CH_3) **0.98** (d, ^3J = 5.8 Hz, CH_3)	-	-
Met	**4.45** (dd, ^3J= 8.9 Hz, ^3J= 4.5 Hz, CH)	**1.97-2.06** (m, CH) **2.15-2.22** (m, CH)	**2.54-2.64** (m, CH_2)	**2.13** (s, CH_3)	-	-

Table 8.10. Chemical shifts of proton resonance for peptide **9** (Chemical shifts in ppm (D_2O)).

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Lys	**4.04** (t, ^3J= 6.5 Hz, CH)	**1.89-1.97** (m, CH_2)	**1.40-1.54** (m, CH_2)	**1.89-1.97** (m, CH_2)	**3.02** (t, ^3J= 7.6 Hz, CH_2)	-
Lys (thioAc)	**4.35-4.45** (m, CH)	**1.89-1.97** (m, CH_2)	**1.40-1.54** (m, CH_2)	**1.76-1.89** (m, CH_2)	**3.63** (t, ^3J= 7.0 Hz, CH_2)	2.52 (s, CH_3)
Leu	**4.35-4.45** (m, CH)	**1.65-1.76** (m, CH_2)	1**.65-1.76** (m, CH)	**0.92** (d, ^3J= 5.8 Hz, CH_3) **0.98** (d, ^3J= 5.9 Hz, CH_3)	-	-

Table 8.11. Chemical shifts of proton resonance for peptide **10 (**Chemical shifts in ppm (D_2O))**.**

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
His	**4.35** (t, ^3J= 6.6 Hz, CH)	**3.44** (d, ^3J= 6.7 Hz, CH)	-	-	-	**7.45** (s, 4'CH) **8.74** (s, 2´CH) (imidazole)
Asp	**4.71-4.76** (m, CH)	**2.73** (dd, ^3J= 16.7 Hz, ^2J= 8.7 Hz, CH) **2.88** (dd, ^3J= 16.7 Hz, ^2J= 5.3 Hz, CH)	-	-	-	-
Lys (thioAc)	**4.41-4.43** (m, CH)	**1.78-1.97** (m, CH_2)	**1.38-1.52** (m, CH_2)	**1.66-1.76** (m, CH_2)	**3.61** (t, ^3J= 7.0 Hz, CH_2)	**2.51** (s, CH_3)
Thr	**4.38** (d, ^3J= 5.4 Hz, CH)	**4.18-4.23** (m, CH)	**1.24** (d, ^3J= 16.7 Hz, CH_3)	-	-	-
Ile	**4.24** (d, ^3J= 6.1 Hz, CH)	**1.89-1.95** (m, CH)	**0.94** (d, ^3J= 6.9 Hz, CH_3) **1.17-1.23** (m, CH) **1.42-1.47** (m, CH)	**0.90** (t, ^3J= 7.4 Hz, CH_3)	-	-

Table 8.12. Chemical shifts of proton resonance for peptide **11 (**Chemical shifts in ppm (D_2O))**.**

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Ser	**4.21** (t, ^3J= 5.9 Hz, CH)	**3.97-4.05** (m, CH_2)	-	-	-	-
Lys	**4.36-4.45** (m, CH)	**1.75-1.91** (m, CH_2)	**1.37-1.53** (m, CH_2)	**1.67-1.75** (m, CH_2)	**3.02** (t, ^3J= 7.0 Hz, CH_2)	-
Lys (thioAc)	**4.36-4.45** (m, CH)	**1.75-1.91** (m, CH_2)	**1.37-1.53** (m, CH_2)	**1.67-1.75** (m, CH_2)	**3.61** (t, ^3J= 7.0 Hz, CH_2)	**2.52** (s, CH3)
Thr	**4.36-4.45** (m, CH)	**4.12-4.31** (m, CH)	**1.23** (d, ^3J= 6.4 Hz, CH_3)	-	-	-
Ile	**4.25** (d, ^3J= 6.2 Hz, CH)	**1.86-2.01** (m, CH)	**0.95** (d, ^3J= 6.8 Hz, CH_3) **1.22-1.28** (m, CH) **1.39-1.54** (m, CH)	**0.92** (t, ^{3}J= 7.4 Hz, CH_3)	-	-

Table 8.13. Chemical shifts of proton resonance for peptide **12 (**Chemical shifts in ppm (D_2O)**).**

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Ser	**4.20** (t, ^3J= 5.0 Hz, CH)	**3.98-4.06** (m, CH_2)	-	-	-	-
Asp	**4.79-4.80** (m, CH)	**2.76** (dd, ^3J= 16.8 Hz, ^2J= 8.4 Hz, CH) **2.88** (dd, ^3J= 16.8 Hz, ^2J= 5.3 Hz, CH)	-	-	-	-
Lys (thioAc)	**4.40** (dd, ^3J= 8.0 Hz, ^3J= 6.3 Hz, CH)	**1.74-1.87** (m, CH_2)	**1.36-1.50** (m, CH_2)	**1.60-1.74** (m, CH_2)	**3.61** (t, ^3J= 7.0 Hz, CH_2)	**2.52** (s, CH_3)
Leu	**4.41-4.44** (m, CH)	**1.61-1.73** (m, CH_2)	**1.61-1.73** (m, CH)	**0.88-0.95** (m, CH_3) **0.97** (d, ^3J= 5.8 Hz, CH_3)	-	-
Ile	**4.21-4-25** (m, CH)	**1.87-1.97** (m, CH)	**0.89-0.97** (m, CH_3) **1.18-1.26** (m, CH) **1.43-1.51** (m, CH)	**0.89-0.97** (m, CH_3)	-	-

Table 8.14. Chemical shifts of proton resonance for peptide **13** (Chemical shifts in ppm (D_2O)**).**

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Ser	**4.13** (7, ^3J= 4.9 Hz, CH)	**4.00** (d, ^3J= 4.9 Hz, CH)	-	-	-	-
Asp	**4.72-4.82** (m, CH)	**2.78** (dd, ^3J= 16.7 Hz, ^2J= 7.7 Hz, CH) **2.88** (dd, ^3J= 16.6 Hz, ^2J= 5.5 Hz, CH)	-	-	-	-
Lys (thioAc)	**4.39-4.44** (m, CH)	**1.74-1.82** (m, CH) **1.86-1.95** (m, CH)	**1.38-1.55** (m, CH_2)	**1.66-1.74** (m, CH_2)	**3.61** (t, ^3J= 7.0 Hz, CH_2)	**2.54** (s, CH_3)
Thr	**4.31** (d, ^3J= 4.9 Hz, CH)	**4.19-4.24** (m, CH)	**1.24** (d, ^3J= 6.4 Hz, CH_3)	-	-	-
Met	**4.37-4.42** (m, CH)	**1.98-2.07** (m, CH) **2.12-2.20** (m, CH)	**2.55-2.64** (m, CH_2)	**2.14** (s, CH_3)	-	-

Table 8.15. Chemical shifts of proton resonance for peptide **14 (**Chemical shifts in ppm (D_2O)**).**

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
His	**4.34-4.38** (m, CH)	**2.97-3.06** (m, CH_2)	-	-	-	**7.45** (s, 4'CH) **8.76** (s, 2´CH) (imidazole)
Lys	**4.38-4.46)** (m, CH)	**1.77-1.92** (m, CH_2)	**1.38-1.54** (m, CH_2)	**1.65-1.77** (m, CH_2)	**3.01** (t, ^3J= 7.6 Hz, CH_2)	-
Lys (thioAc)	**4.38-4.46)** (m, CH)	**1.77-1.92** (m, CH_2)	**1.38-1.54** (m, CH_2)	**1.65-1.77** (m, CH_2)	**3.55-3.67** (m, CH_2)	**2.52** (s, CH_3)
Thr	**4.38-4.46)** (m, CH)	**4.16-4.23** (m, CH)	**1.24** (d, ^3J= 6.4 Hz, CH_3)	-	-	-
Ile	**4.30-4.35** (m, CH)	**1.93-2.01** (m, CH)	**0.97** (d, ^3J= 6.8 Hz, CH_3) **1.22-1.31** (m, CH) **1.42-1.51** (m, CH)	**0.92** (t, ^3J= 7.4 Hz, CH_3)	-	-

Table 8.16. Chemical shifts of proton resonance for peptide **15 (**Chemical shifts in ppm (D_2O)**).**

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Ser	**4.20** (t, ^3J= 5.0 Hz, CH)	**4.00-4.04** (m, CH_2)	-	-	-	-
Asp	**4.76-4.80** (m, CH)	**2.78** (dd, ^3J= 16.7 Hz, ^2J= 8.2 Hz, CH) **2.89** (dd, ^3J= 16.6 Hz, ^2J= 5.3 Hz, CH)	-	-	-	-
Lys (thioAc)	**4.29-4.34** (m, CH)	**1.74-1.90** (m, CH_2)	**1.36-1.48** (m, CH_2)	**1.59-1.74** (m, CH_2)	**3.62** (t, ^3J= 7.0 Hz, CH_2)	**2.53** (s, CH_3)
Leu	**4.37-4.41** (m, CH)	**1.62-1.69** (m, CH_2)	**1.62-1.69** (m, CH)	**0.91** (d, ^3J= 5.7 Hz, CH_3) **0.97** (d, ^3J= 5.7 Hz, CH_3)	-	-
Met	**4.44** (dd, ^3J= 9.0 Hz, ^2J= 4.5 Hz, CH)	**1.97-2.06** (m, CH) **2.14-2.21** (m, CH)	**2.48-2.64** (m, CH_2)	**2.12** (s, CH_3)	-	-

Table 8.17. Chemical shifts of proton resonance for peptide **16 (**Chemical shifts in ppm (D_2O)).

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Ala	**4.11** (q, ^3J= 7.1 Hz, CH)	**1.56** (d, ^3J= 7.1 Hz, CH_3)	-	-	-	-
Asp	**4.70-4.77** (m, CH)	**2.74** (dd, ^3J= 16.6 Hz, ^2J= 8.5 Hz, CH) **2.89** (dd, ^3J= 16.6 Hz, ^2J= 5.7 Hz, CH)	-	-	-	-
Lys (thioAc)	**4.40-4.44** (m, CH)	**1.76-1.85** (m, CH) **1.86-1.97** (m, CH)	**1.39-1.48** (m, CH_2)	**1.67-1.75** (m, CH_2)	**3.61** (t, ^3J= 7.1 Hz, CH_2)	**2.52** (s, CH_3)
Thr	**4.37** (d, ^3J= 5.5 Hz, CH)	**4.17-4.23** (m, CH)	**1.24** (d, ^3J= 6.4 Hz, CH_3)	-	-	-
Ile	**4.25** (d, ^3J= 6.1 Hz, CH)	**1.87-1.98** (m, CH)	**0.95** (d, ^3J= 6.9 Hz, CH_3) **1.18-1.28** (m, CH) **1.43-1.51** (m, CH)	**0.92** (t, ^3J= 7.4 Hz, CH_3)	-	-

Table 8.18. Chemical shifts of proton resonance for peptide **17 (**Chemical shifts in ppm (D_2O)).

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Ser	**4.16-4.20** (m, CH)	**3.98-4.07** (m, CH_2)	-	-	-	-
Ala	**4.43** (q, ^3J= 7.7 Hz, CH)	**1.44** (d, ^3J= 7.2 Hz, CH_3)	-	-	-	-
Lys (thioAc)	**4.37-4.41** (m, CH)	**1.76-1.84** (m, CH) **1.84-1.93** (m, CH)	**1.38-1.52** (m, CH_2)	**1.71** (q, ^3J= 7.3 Hz, CH_2)	**3.62** (t, ^3J= 7.0 Hz, CH_2)	**2.52** (s, CH_3)
Thr	**4.37-4.41** (m, CH)	**4.21-4.25** (m, CH)	**1.22** (d, ^3J= 6.4 Hz, CH_3)	-	-	-
Ile	**4.16-4.20** (m, CH)	**1.84-1.94** (m, CH)	**0.93** (d, ^3J= 7.0 Hz, CH_3) **1.14-1.26** (m, CH) **1.39-1.54** (m, CH)	**0.91** (t, ^3J= 7.5 Hz, CH_3)	-	-

Table 8.19. Chemical shifts of proton resonance for peptide **18 (**Chemical shifts in ppm (D_2O)).

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Ser	**4.20** (t, ^3J= 4.9 Hz, CH)	**3.98-4.06** (m, CH_2)	-	-	-	-
Asp	**4.72-4.79** (m, CH)	**2.78** (dd, ^3J= 16.7 Hz, ^2J= 8.2 Hz, CH) **2.90** (dd, ^3J= 16.7 Hz, ^2J= 5.4 Hz, CH)	-	-	-	-
Lys (thioAc)	**4.28-4.34** (m, CH)	**1.74-1.81** (m, CH) **1.81-1.89** (m, CH)	**1.42-1.52** (m, CH_2)	**1.70** (q, ^3J= 7.2 Hz, CH_2)	**3.61** (t, ^3J= 7.0 Hz, CH_2)	**2.52** (s, CH_3)
Ala	**4.40** (q, ^3J= 7.1 Hz, CH)	**1.41** (d, ^3J= 7.2 Hz, CH_3)	-	-	-	-
Ile	**4.22** (d, ^3J= 6.0 Hz, CH)	**1.89-1.96** (m, CH)	**0.95** (d, ^3J= 6.9 Hz, CH_3) **1.16-1.28** (m, CH) **1.41-1.53** (m, CH)	**0.92** (t, ^3J= 7.4 Hz, CH_3)	-	-

Table 8.20. Chemical shifts of proton resonance for peptide **19 (**Chemical shifts in ppm (D_2O)**).**

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Ser	**4.21** (t, ^3J= 4.9 Hz, CH)	**4.01-4.05** (m, CH_2)	-	-	-	-
Asp	**4.74-4.80** (m, CH)	**2.85** (dd, ^3J= 16.9 Hz, ^2J= 7.8 Hz, CH) **2.95** (dd, ^3J= 16.9 Hz, ^2J= 5.6 Hz, CH)	-	-	-	-
Lys (thioAc)	**4.37-4.42** (m, CH)	**1.76-1.85** (m, CH) **1.85-1.95** (m, CH)	**1.36-1.51** (m, CH_2)	**1.70** (q, ^3J= 7.3 Hz, CH_2)	**3.62** (t, ^3J= 7.0 Hz, CH_2)	**2.52** (s, CH_3)
Thr	**4.31-4.37** (m, CH)	**4.20-4.27** (m, CH)	**1.25** (d, ^3J= 6.4 Hz, CH_3)	-	-	-
Ala	**4.31-4.39** (m, CH)	**1.44** (d, ^3J= 7.3 Hz, CH_3)	-	-	-	-

Table 8.21. Chemical shifts of proton resonance for peptide **20 (**Chemical shifts in ppm (D_2O)**).**

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
Ala	**4.12** (q, ^3J= 7.1 Hz, CH)	**1.55** (d, ^3J= 7.1 Hz, CH_3)	-	-	-	-
Lys	**4.34-4.42** (m, CH)	**1.76-1.89** (m, CH_2)	**1.36-1.50** (m, CH_2)	**1.61-1.76** (m, CH_2)	**3.02** (t, ^3J= 7.6 Hz, CH_2)	-
Lys (thioAc)	**4.34-4.42** (m, CH)	**1.76-1.89** (m, CH_2)	**1.36-1.50** (m, CH_2)	**1.61-1.76** (m, CH_2)	**3.63** (t, ^3J= 7.0 Hz, CH_2)	**2.53** (s, CH_3)
Leu	**4.36-4.43** (m, CH)	**1.61-1.71** (m, CH_2)	**1.61-1.71** (m, CH)	**0.91** (d, ^3J= 5.8 Hz, CH_3) **0.97** (d, ^3J= 5.8 Hz, CH_3)	-	-
Met	**4.30-4.35** (m, CH)	**1.95-2.04** (m, CH) **2.10-2.21** (m, CH)	**2.48-2.62** (m, CH_2)	**2.12** (s, CH_3)	-	-

Table 8.22. Chemical shifts of proton resonance for peptide **21 (**Chemical shifts in ppm (D_2O)**).**

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
His	**4.28** (t, ^3J= 6.3 Hz, CH)	**3.36** (d, ^3J= 6.4 Hz, CH_3)	-	-	-	**7.36** (s, 4'CH) **8.44** (s, 2´CH) (imidazole)
Ala	**4.41-4.45** (m, CH)	**1.43** (d, ^3J= 7.2 Hz, CH_3)	-	-	-	-
Lys (thioAc)	**4.32-4.36** (m, CH)	**1.78-1.90** (m, CH_2)	**1.38-1.51** (m, CH_2)	**1.68-1.75** (m, CH_2)	**3.62** (t, ^3J= 7.0 Hz, CH_2)	**2.52** (s, CH_3)
Leu	**4.39-4.42** (m, CH)	**1.63-1.70** (m, CH_2)	**1.63-1.70** (m, CH)	**0.90** (d, ^3J= 5.8 Hz, CH_3) **0.95** (d, ^3J= 5.8 Hz, CH_3)	-	-
Met	**4.29-4.33** (m, CH)	**1.92-2.01** (m, CH) **2.08-2.17** (m, CH)	**2.46-2.58** (m, CH_2)	**2.11** (s, CH_3)	-	-

Table 8.23. Chemical shifts of proton resonance for peptide **22 (**Chemical shifts in ppm (D_2O)**).**

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
His	**4.33-4.37** (m, CH)	**3.39-3.48** (m, CH_2)	-	-	-	**7.46** (s, 4'CH) **8.76** (s, 2´CH) (imidazole)
Lys	**4.31-4.35** (m, CH)	**1.76-1.90** (m, CH_2)	**1.43-1.53** (m, CH_2)	**1.72** (q, ^3J= 7.3 Hz, CH_2)	**3.02** (t, ^3J= 7.6 Hz, CH_2)	-
Lys (thioAc)	**4.37-4.42** (m, CH)	**1.76-1.90** (m, CH_2)	**1.43-1.53** (m, CH_2)	**1.72** (q, ^3J= 7.3 Hz, CH_2)	**3.58-3.67** (m, CH_2)	**2.52** (s, CH_3)
Ala	**4.34-4.40** (m, CH)	**1.43** (d, ^3J= 7.2 Hz, CH_3)	-	-	-	-
Met	**4.54** (dd, ^3J= 9.5 Hz, ^2J= 4.6 Hz, CH)	**1.98-2.08** (m, CH) **2.17-2.25** (m, CH)	**2.54-2.60** (m, CH) **2.62-2.69** (m, CH)	**2.13** (s, CH_3)	-	-

Table 8.24. Chemical shifts of proton resonance for peptide **23 (**Chemical shifts in ppm (D_2O)**).**

Residue	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$	Others
His	**4.34-4.39** (m, CH)	**3.43-3.47** (m, CH_2)	-	-	-	**7.46** (s, 4'CH) **8.75** (s, 2´CH) (imidazole)
Lys	**4.30-4.42** (m, CH)	**1.77-1.90** (m, CH_2)	**1.44-1.52** (m, CH_2)	**1.62-1.76** (m, CH_2)	**3.02** (t, ^3J= 7.6 Hz, CH_2)	-
Lys (thioAc)	**4.30-4.42** (m, CH)	**1.77-1.90** (m, CH_2)	**1.44-1.52** (m, CH_2)	**1.62-1.76** (m, CH_2)	**3.60-3.65** (m, CH_2)	**2.52** (s, CH_3)
Leu	**4.38-4.42** (m, CH)	**1.62-1.69** (m, CH_2)	**1.62-1.69** (m, CH)	**0.91** (d, ^3J= 6.1 Hz, CH_3) **0.96** (d, ^3J= 6.0 Hz, CH_3)	-	-
Ala	**4.29-4.35** (m, CH)	**1.42** (d, ^3J= 7.3 Hz, CH_3)	-	-	-	-

Ser-Asp-Lys(ε-thioAc)-Thr-Ile (1). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 12.32, 16.64, 20.35, 23.77, 25.96, 27.75, 32.01, 33.75, 37.96, 38.18, 47.28, 52.13, 54.93, 55.68, 59.66, 60.40, 61.46, 68.23, 168.84, 172.27, 173.46, 174.77, 176.16, 176.94, 201.32. ESI-MS (m/z): 621.2 $[M + H]^+$.

Ser-Asp-Lys(ε-thioAc)-Thr (2). ^{13}C NMR (DMSO-*d6*/$CDCl_3$, $CDCl_3$ as an internal standard at 79.16 ppm) δ = 20.29, 23.01, 27.06, 31.61, 33.01, 37.14, 45.60, 50.25, 52.86, 54.92, 58.21, 61.40, 66.50, 168.18, 170.51, 171.68, 172.18, 172.42, 198.99. ESI-MS (m/z): 508.2 $[M + H]^+$.

Asp-Lys(ε-thioAc)-Thr-Ile (3). ^{13}C NMR (DMSO-*d6*/$CDCl_3$, $CDCl_3$ as an internal standard at 79.16 ppm) δ = 11.60, 15.69, 20.08, 23.07, 24.85, 27.14, 31.75, 33.00, 36.82, 37.41, 45.56, 50.54, 53.18, 56.97, 58.94, 66.60, 169.91, 170.03, 171.56, 172.37, 172.97, 199.01. ESI-MS (m/z): 534.2 $[M + H]^+$.

Asp-Lys(ε-thioAc)-Thr (4). ^{13}C NMR (DMSO-*d6*/$CDCl_3$, $CDCl_3$ as an internal standard at 79.16 ppm) δ = 20.56, 23.10, 27.20, 31.73, 33.08, 36.77, 45.69, 49.96, 53.08, 58.25, 66.55, 168.89, 171.86, 171.91, 172.31, 199.20. ESI-MS (m/z): 421.2 $[M + H]^+$.

Ser-Asp-Lys(ε-thioAc) (5). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 22.06 (Acetate CH_3), 23.88, 27.88, 32.15, 33.79, 37.90, 47.61, 52.21, 55.45, 55.96, 61.62, 169.15, 173.22, 176.44, 178.35, 201.62. ESI-MS (m/z): 407.2 $[M + H]^+$.

His-Lys-Lys(ε-thioAc)-Leu-Met (6). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 15.67, 22.32, 23.44, 23.67, 23.94, 25.79, 27.77, 27.88, 27.96, 30.98, 32.09 (2xCH_2), 32.40, 33.83, 40.60, 40.98, 47.46, 53.21, 53.83, 54.93, 55.17, 55.37, 117.84 (TFA CF_3, q, $^1J_{C,F}$ = 292 Hz), 120.13, 127.27, 135.98, 169.47, 174.59, 174.81, 174.87, 178.53, 201.67. ESI-MS (m/z): 714.4 $[M + H]^+$.

His-Lys-Lys(ε-thioAc)-Leu (7). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 22.09, 23.36, 23.68, 23.75, 25.89, 27.59, 27.83, 27.93, 32.00, 32.06, 33.81, 40.55, 40.77, 47.40, 52.87, 53.10, 55.14, 55.20, 117.83 (TFA CF_3, q, $^1J_{C,F}$ = 293 Hz), 120.16, 127.03, 135.84, 169.29, 174.50, 174.88, 177.62, 201.66. ESI-MS (m/z): 583.4 $[M + H]^+$.

Lys-Lys(ε-thioAc)-Leu-Met (8). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 15.66, 22.42, 22.56, 23.60, 23.85, 25.74, 27.83, 27.96, 30.92, 31.88, 32.01, 32.14, 33.83, 40.45, 41.03, 47.44, 53.82, 54.15 (2xCH), 55.24, 117.83 (TFA CF_3, q, $^1J_{C,F}$ = 293 Hz), 170.77, 174.52, 175.05, 177.72, 201.65. ESI-MS (m/z): 577.4 $[M + H]^+$.

Lys-Lys(ε-thioAc)-Leu (9). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 22.10, 22.48 (2xCH_2), 23.68, 25.88, 27.82, 27.93, 31.88, 32.00, 33.82, 40.44, 40.72, 47.39, 52.91, 54.14, 55.18, 170.75, 174.76, 177.56, 201.69. ESI-MS (m/z): 446.4 $[M + H]^+$.

His-Asp-Lys(ε-thioAc)-Thr-Ile (10). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 12.23, 16.63, 20.35, 23.90, 26.10, 27.56, 27.91, 32.00, 33.80, 38.11, 38.69, 47.47, 52.54, 53.25, 55.41, 60.19, 60.64, 68.51, 120.04, 127.18, 135.99, 169.27, 172.58, 173.98, 175.19, 176.86, 177.86, 201.66. ESI-MS (m/z): 671.3 $[M + H]^+$.

Ser-Lys-Lys(ε-thioAc)-Thr-Ile (11). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 12.24, 16.64, 20.30, 23.41, 23.86, 26.12, 27.81, 27.86, 32.03, 32.15, 33.81, 38.06, 40.56, 47.43, 55.15, 55.16, 55.89, 60.05, 60.37, 61.68, 68.54, 117.83 (TFA CF_3, q, $^1J_{C,F}$ = 293 Hz), 169.15, 172.54, 174.69, 175.25, 177.66, 201.65. ESI-MS (m/z): 634.4 $[M + H]^+$.

Ser-Asp-Lys(ε-thioAc)-Leu-Ile (12). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 12.20, 16.62, 22.35, 23.65, 23.83, 25.77, 26.07, 27.87, 32.08, 33.82, 38.19, 38.33, 40.90, 47.50, 52.27, 53.76, 55.17, 55.90, 59.99, 61.59, 169.10, 173.67, 174.77, 175.13, 176.75, 177.81, 201.66. ESI-MS (m/z): 633.4 $[M + H]^+$.

Ser-Asp-Lys(ε-thioAc)-Thr-Met (13). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 15.57, 20.40, 23.67, 27.63, 30.62, 31.88, 31.99, 33.66, 37.92, 46.93, 51.75, 53.76, 54.60, 55.35, 60.19, 61.26, 67.84, 168.43, 171.78, 172.94, 174.14, 175.21, 176.27, 200.87. ESI-MS (m/z): 639.2 $[M + H]^+$.

His-Lys-Lys(ε-thioAc)-Thr-Ile (14). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 12.15, 16.53, 20.36, 23.40, 23.87, 26.14, 27.59, 27.83, 27.95, 32.08, 32.13, 33.81, 37.84, 40.56, 47.41, 53.14, 55.16, 55.32, 59.14,

60.37, 68.57, 117.84 (TFA CF_3, q, $^1J_{C,F}$ = 293 Hz), 120.13, 127.02, 135.89, 169.31, 172.78, 174.69, 175.16, 176.61, 201.69. ESI-MS (m/z): 684.4 $[M + H]^+$.

Ser-Asp-Lys(ε-thioAc)-Leu-Met (15). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 15.67, 22.31, 23.64, 23.83, 25.77, 27.90, 30.95, 32.04, 32.06, 33.83, 38.16, 40.89, 47.54, 52.20, 53.80, 54.25, 55.24, 55.90, 61.61, 169.13, 173.66, 174.80, 175.22, 176.60, 177.87, 201.66. ESI-MS (m/z): 651.3 $[M + H]^+$.

Ala-Asp-Lys(ε-thioAc)-Thr-Ile (16). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 12.25, 16.63, 17.99, 20.32, 23.79, 26.07, 27.83, 32.10, 33.79, 38.06, 38.26, 47.47, 50.31, 52.18, 55.04, 59.97, 60.59, 68.43, 171.92, 172.54, 173.74, 175.09, 176.53, 177.54, 201.59. ESI-MS (m/z): 605.3 $[M + H]^+$.

Ser-Ala-Lys(ε-thioAc)-Thr-Ile (17). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 12.34, 16.76, 18.11, 20.26, 23.89, 26.07, 27.86, 32.15, 33.80, 38.36, 47.45, 51.19, 55.10, 55.93, 60.34, 61.03, 61.66, 68.54, 168.98, 172.24, 175.32, 175.87, 178.80, 201.63. ESI-MS (m/z): 577.3 $[M + H]^+$.

Ser-Asp-Lys(ε-thioAc)-Ala-Ile (18). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 12.24, 16.64, 17.86, 23.81, 26.07, 27.86, 32.13, 33.81, 38.12, 38.16, 47.51, 50.96, 52.24, 55.07, 55.91, 60.03, 61.61, 169.16, 173.72, 174.65, 175.63, 176.68, 177.99, 201.68. ESI-MS (m/z): 591.3 $[M + H]^+$.

Ser-Asp-Lys(ε-thioAc)-Thr-Ala (19). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 18.04, 20.28, 23.83, 27.83, 31.98, 33.80, 37.50, 47.48, 50.77, 51.96, 55.37, 55.90, 60.45, 61.64, 68.52, 169.18, 172.47, 173.66, 175.32, 175.96, 178.41, 201.67. ESI-MS (m/z): 579.2 $[M + H]^+$.

Ala-Lys-Lys(ε-thioAc)-Leu-Met (20). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 15.70, 18.09, 22.34, 23.47, 23.68, 23.86, 25.76, 27.84, 27.90, 30.97, 32.02, 32.18, 32.41, 33.83, 40.61, 40.99, 47.48, 50.29, 53.82, 54.88, 55.01, 55.02, 172.04, 174.57, 174.78, 174.88,178.43, 201.63. ESI-MS (m/z): 648.4 $[M + H]^+$.

His-Ala-Lys(ε-thioAc)-Leu-Met (21). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 15.69, 18.11, 22.27, 23.71, 23.95, 25.80, 27.93, 28.68, 31.00, 32.07, 32.78, 33.82, 40.93, 47.49, 51.08, 53.64, 53.86, 55.31, 55.51,

119.71, 128.95, 136.52, 170.11, 174.70, 174.94, 175.90, 179.11, 201.65. ESI-MS (m/z): 657.4 $[M + H]^+$.

His-Lys-Lys(ε-thioAc)-Ala-Met (22). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 15.59, 17.99, 21.92 (Acetate CH_3), 23.41, 23.84, 27.61, 27.83, 27.98, 30.88, 31.57, 32.08, 32.18, 33.82, 40.56, 47.43, 50.94, 53.14, 53.38, 55.16 (2xCH), 117.83 (TFA CF_3, q, $^1J_{C,F}$ = 293 Hz), 120.13, 127.04, 135.90, 169.31, 174.59, 174.65, 175.75, 177.02, 201.66. ESI-MS (m/z): 672.3 $[M + H]^+$.

His-Lys-Lys(ε-thioAc)-Leu-Ala (23). ^{13}C NMR (D_2O/DMSO-*d6*, DMSO as an internal standard at 39.39 ppm) δ = 18.07, 22.31, 23.41, 23.64, 23.85, 25.72, 27.61, 27.83, 27.92, 31.99, 32.06, 33.80, 40.55, 41.23, 47.41, 50.60, 53.11, 53.54, 55.13, 55.28, 117.83 (TFA CF_3, q, $^1J_{C,F}$ = 292 Hz), 120.16, 127.04, 135.86, 164.20 (TFA C=O, q, $^2J_{C,F}$ = 35 Hz), 169.32, 174.57, 174.84, 175.05, 178.34, 201.65. ESI-MS (m/z): 654.4 $[M + H]^+$.

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9 GENERAL DISCUSSION AND CONCLUSIONS

9.1 General discussion

SIRT2 was reported the first time in 1999 as one of the five human sirtuins characterized by that time (Frye 1999). Research around SIRT2 has rapidly increased since then, and SIRT2 together with SIRT1 are the most studied of the human sirtuins. However, the biological function of SIRT2 appears still to be largely unknown. In the beginning of this study, the only known SIRT2 inhibitors were sirtinol and A3, and their reported inhibitory activities were 38 μM and 45 μM, respectively (Grozinger *et al.* 2001). There was a clear need for new types of potent inhibitors for SIRT2 to offer tools for biological research.

Experts in molecular modelling methods in our research group started the SIRT project by virtual screening of commercial compound databases for new types of SIRT2 inhibitors. Characterization of the binding and interaction properties of SIRT2 inhibitors was an important step in this process. However, the task was challenging and, probably, bigger than it was though to be in the beginning. There was only one experimental crystal structure of SIRT2 publicly available and this apo-form did not provide any experimental data on the binding sites and modes of the inhibitors (Finnin *et al.* 2001).

The first search in the database from Maybridge (Maybridge Chemical Company Ltd) provided two related hit compounds (Tervo *et al.* 2004), which were used to design a new backbone for SIRT2 inhibitors. Three series of *N,N′*-bisbenzylidenebenzene-1,4-diamine and *N,N′*-bisbenzylidenenaphthalene-1,4-diamine derivatives were synthesized and tested in the radioactive [^{3}H]-substrate based deacetylation assay for SIRT2 (Chapter 5).

The series of potent compounds proved that the new backbone could be used for designing new SIRT2 inhibitors. The most potent compound, *N,N′*-bis(2-hydroxybenzylidene)benzene-1,4-diamine, was equipotent with the most potent hit compound and sirtinol. Also, the new compounds were able to adopt binding modes that share similarities with the best ranked binding conformation of sirtinol. However, there were several reasons why the series of this new backbone were not continued. The synthesized compounds had a low solubility in water and also in DMSO. In addition,

the optic properties of the compounds disturbed the use of the fluorescence based assay and the compounds had to be tested with a time-consuming radioactivity based assay. Furthermore, the preliminary results from toxicity studies in Neuro-2a neuroblastoma cells suggested that the hit structures were toxic to cells at the concentration of 30 μM (unpublished results). Otherwise, the series would have been continued with unsymmetrical compounds as there was no requirement of symmetry in the binding site.

The second search in the database from LeadQuest (Tripos Associates) provided the interesting hit structure with the *N*-(3-(4-hydroxyphenyl)-propenoyl)-amino acid tryptamide backbone (Tervo *et al.* 2006). However, as the molecular weight of the hit compound was relatively large, over 500 g/mol, it was reasonable to decrease the molecular size and look for the essential parts of the hit structure for the inhibitory activity (Chapter 6). As a result, the molecular size of the hit compound could drastically be decreased without loosing the inhibitory activity. The new compounds were also more drug-like than the compounds developed from the first database search. Although our research is in an early stage considering the whole drug development process, the drug-likeness of the compounds should also be considered.

The research of this new interesting backbone was continued and the structure-activity relationships were studied by different replacements in the original hit structure (Chapter 7). Unfortunately, the inhibitory activities could not be improved. However, the complete series of the compounds with the *N*-(3-phenylpropenoyl)-glycine tryptamide backbone and the series of the compounds with the smaller 3-phenylpropenoic acid tryptamide backbone provided tools to molecular modellers to analyze binding modes of the compounds with molecular modelling methods.

The SIRT2 activity with the hit compounds **1** and **2** found by molecular modeling and virtual screening (Tervo *et al.* 2004, 2006) and synthesized compounds **3–5** (figure 9.1) has also been tested using acetylated α-tubulin as the substrate (figure 9.2). Before the enzymatic reaction, acetylated α-tubulin shows an intensive band at 0 hour and after the enzymatic reaction the amount of acetylated α-tubulin is barely visible with SIRT2 at five hours. With compounds **1** and **2** the SIRT2 inhibitory activity was clearly noticed at a concentration of 200 μM. On the other hand, for compounds **3–5** the amount of acetylated α-tubulin seems to be identical to the spot after the uninhibited enzymatic

reaction at five hours (unpublished results). However, these are preliminary results and the analyses shows only the inhibitory potency of compounds for other substrates than used in the assays for SIRT2 activity described in Chapter 4.4.

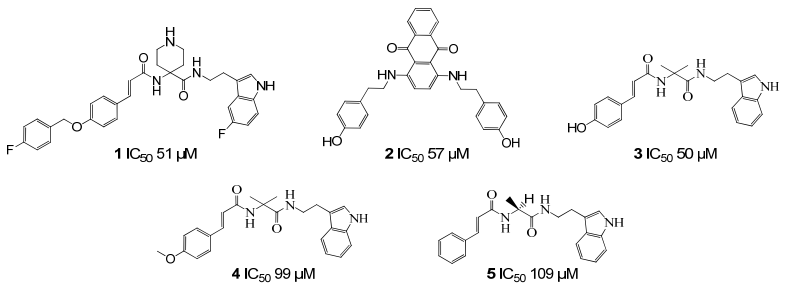

Figure 9.1. Compounds **1**, **2**, **3**, **4**, and **5**.

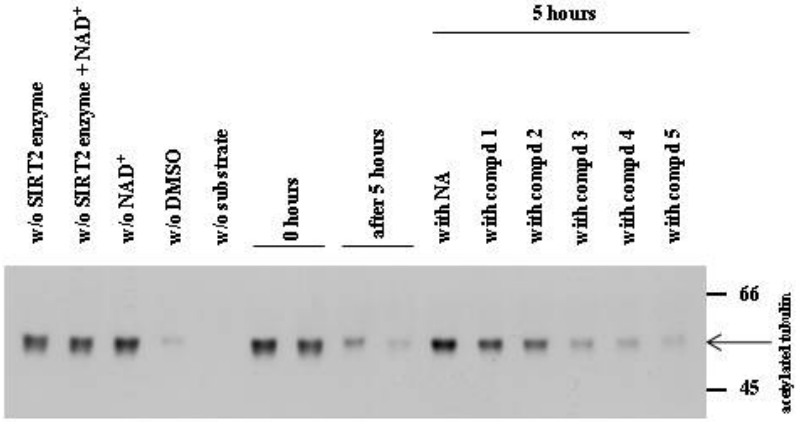

Figure 9.2. Western plot analyses of compounds **1**, **2**, **3**, **4**, and **5**.

Since the exact binding site of the SIRT2 inhibitors is not fully characterized and the binding mode analyses did not provide new information to continue with the studied inhibitor backbones, a new approach using the peptide sequences from the substrate peptides for designing SIRT2 inhibitors was examined. The binding site of the substrates (the active site) offers more exact information about the interaction properties between the substrate and the enzyme. Fatkins *et al.* (2006) showed that an 18-mer peptide based on the p53 protein sequence containing N^{ε}-thioacetyl-lysine inhibited

SIRT1 on a low micromolar level. The result can be considered as a starting point for the development of substrate-based SIRT2 and SIRT1 inhibitors.

Both p53 and α-tubulin based sequences were studied (Chapter 8). Not only new synthetic methods of inhibitors using a peptide synthesizer and solid phase chemistry, but also the encouraging results from the *in vitro* tests, brought new enthusiasm to the project. The first time ever published potent tri-, tetra-, and pentapeptides provide a promising starting point for the development of small peptidomimetic SIRT1 and SIRT2 inhibitors. In addition, the found selectivity for SIRT1 over SIRT2 was extremely interesting.

The amount of open questions and disbelief in reaching a nanomolar inhibitory activity for SIRT2 reflect the challenges that exist in the research of SIRT2 inhibitors. However, the research is team work and it is a privilege to work with scientists with expertise in different fields and reach the results that take the research of SIRT2 inhibitors forward.

9.2 Conclusions

The present study describes the design, synthesis, characterization and *in vitro* evaluation of novel SIRT2 inhibitors. The following conclusions can be made from the present study.

1. The new different backbones of SIRT2 inhibitors were used to synthesize new series of SIRT2 inhibitors, which overcame many of the problems associated with the first known SIRT2 inhibitors.

2. The modified new compounds indicated the value of the backbones for the design of SIRT2 inhibitors. Several potent new SIRT2 inhibitors were synthesized and tested *in vitro* for SIRT2. The new compounds revealed the structure-activity relationships and gave important information for molecular modelling studies.

3. The short peptide sequences indicated the value of the known SIRT2

substrates for design of SIRT2 and SIRT1 substrate-based inhibitors. A series of tri-, tetra-, and pentapeptides were the shortest sequences and among the most potent SIRT2 and SIRT1 inhibitors published so far.

4. Weak SIRT2 selectivity over SIRT1 was shown with the most potent compounds of *N*-(3-(4-hydroxyphenyl)-propenoyl)-amino acid tryptamides. Clear SIRT1 selectivity over SIRT2 was shown with two of the p53 based tri- and tetrapeptides, which were more selective than well-known selective SIRT1 inhibitor EX-527.

In summary, this doctoral dissertation has introduced several new SIRT2 inhibitor backbones that interact with different binding sites of the enzyme. The novel SIRT2 inhibitors have already been used in co-operation with molecular modellers and cell biologists for improving the knowledge of the function and the meaning of SIRT2.

9.3 Future perspectives

The biggest barrier in the research of SIRT2 inhibitors is the lack of the SIRT2 crystal structure which would be complexed with the NAD^+, a substrate and/ or a potent inhibitor. Despite the intensive efforts, the work has not succeeded yet. An increased structural diversity of SIRT2 inhibitors will also provide more information for the molecular modellers in their studies of the binding sites.

The substrate-based inhibitors are the most promising lead structures at the moment. The binding site of these inhibitors should be easier to study as they are likely to bind to the substrate binding site of the enzyme. The design of peptidomimetics will be a major objective for the further development of these inhibitors. In addition, it is challenging to study the selectivity between SIRT1 and SIRT2 and what interaction properties are important for the selectivity. The different cell culture studies and the *in vivo* data would provide important biological information of the inhibitors. The collaboration with scientists in different fields will exploit the dynamic screening systems and test assays the best and give the long-term results for the SIRT research.

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