Modulating Sirtuin Activity Design, Synthesis and Evaluation of Sirtuin 2 Inhibitors

TINA SEIFERT



UNIVERSITY OF GOTHENBURG

Department of Chemistry and Molecular Biology University of Gothenburg 2014

DOCTORAL THESIS

Submitted for fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry

Modulating Sirtuin Activity Design, Synthesis and Evaluation of Sirtuin 2 Inhibitors

TINA SEIFERT

Cover illustration: The chroman-4-one scaffold and a potent SIRT2 inhibitor in its binding site in SIRT2.

© Tina Seifert ISBN: 978-91-628-9249-4 http://hdl.handle.net/2077/37294

Department of Chemistry and Molecular Biology SE-412 96 Göteborg Sweden

Printed by Ineko AB Kållered, 2014

To my family

Abstract

Sirtuins (SIRTs) are NAD⁺-dependent lysine deacetylating enzymes targeting histones and a multitude of non-histone proteins. The SIRTs have been related to important cellular processes such as gene expression, cell proliferation, apoptosis and metabolism. They are proposed to be involved in the pathogenesis of e.g. cancer, neurodegeneration, diabetes and cardiovascular disorders. Thus, development of SIRT modulators has attracted an increased interest in recent years.

This thesis describes the design and synthesis of tri- and tetrasubstituted chroman-4one and chromone derivatives as novel SIRT inhibitors. The chroman-4-ones have been synthesized via a one-pot procedure previously developed by our group. Further modifications of the chroman-4-ones using different synthetic strategies have increased the diversity of the substitution pattern. Chromones have been synthesized from the corresponding chroman-4-one precursors. Biological evaluation of these compounds has identified highly selective and potent SIRT2 inhibitors with IC₅₀ values in the low μ M range. Evaluation of selected compounds in cancer cell lines has shown an antiproliferative effect in breast cancer and lung carcinoma cells and an effect on the viability and morphology of brain tumor cells. A binding site for the SIRT2 inhibitors, i.e. the C-pocket of the NAD⁺ binding site, has been proposed using molecular modeling that showed to be consistent with the structure-activity relationship data.

The proposed binding site has been further investigated using a photoaffinity labeling approach. For this, two photoactivatable chroman-4-ones containing either an azide or a diazirine moiety have been synthesized. The diazirine analog was a potent SIRT2 inhibitor. The light-induced incorporation of this photoprobe into SIRT2 followed by mass spectral analysis of the adducts has indicated that a stretch of eight amino acids has been labelled. The amino acids are located around the active site of SIRT2. One of the amino acids is a conserved histidine residue that is positioned at the part of the C-pocket to which the chroman-4-ones presumably bind. However, the low cross-linking yield has complicated the identification of the specific amino acid(s) modified by the probe.

The chroman-4-one scaffold has also been replaced with different analogous bicyclic frameworks, e.g. quinolones, saccharins and benzothiadiazine-1,1-dioxides. Most of the new compounds were less active than the chroman-4-one based inhibitors, but some were moderately potent. Interestingly, the new compounds also possessed moderate SIRT3 inhibitory activity. Thus, cyclic sulfonamides show potential as SIRT2 inhibitors and might also be valuable for the development of SIRT3 selective inhibitors

Keywords: Sirtuin, SIRT2, Inhibitors, Chroman-4-ones, Chromones, Benzothiadiazine-1,1dioxides, Saccharin, Scaffold, Structure-activity relationship, Antiproliferative properties, Binding site, Homology modeling, Photoaffinity labeling, Diazirine, Mass spectrometry.

List of Publications

This thesis is based on the following publications and manuscripts, which are referred to in the text by the Roman numerals I–IV. Paper I is reprinted with kind permission from the publisher.

- I Synthesis and Evaluation of Substituted Chroman-4-one and Chromone Derivatives as Sirtuin 2-Selective Inhibitors
 Maria Fridén-Saxin,* Tina Seifert,* Marie Rydén Landergren, Tiina Suuronen, Maija Lahtela-Kakkonen, Elina M. Jarho, Kristina Luthman Journal of Medicinal Chemistry 2012, 55, 7104–7113.
- II Chroman-4-one- and Chromone-based Sirtuin 2 Inhibitors with Antiproliferative Properties in Cancer Cells
 Tina Seifert, Marcus Malo, Tarja Kokkola, Karin Engen, Maria Fridén-Saxin, Erik A.
 A. Wallén, Maija Lahtela-Kakkonen, Elina M. Jarho, Kristina Luthman
 Accepted for publication in Journal of Medicinal Chemistry
- III Identification of the Binding Site of Chroman-4-one based Sirtuin 2-selective Inhibitors by Photoaffinity Labeling in Combination with Mass Spectrometry Tina Seifert, Marcus Malo, Johan Lengqvist, Carina Sihlbom, Elina M. Jarho, Kristina Luthman Manuscript
- IV Using a Scaffold Replacement Approach towards new Sirtuin Inhibitors
 Tina Seifert, Marcus Malo, Tarja Kokkola, Johanna Steén, Kristian Meinander, Erik
 A. A. Wallén, Elina M. Jarho, Kristina Luthman
 Manuscript

Publications not included in this thesis:

KHMDS Enhanced SmI₂-Mediated Reformatsky Type α-Cyanation Tobias Ankner, Maria Fridén-Saxin, Nils Pemberton, Tina Seifert, Morten Grøtli, Kristina Luthman, Göran Hilmersson

Organic Letters 2010, 12, 2210–2213.

Proline-mediated Formation of Novel Chroman-4-one Tetrahydropyrimidines Maria Fridén-Saxin, Tina Seifert, Lars Kristian Hansen, Morten Grøtli, Mate Erdelyi, Kristina Luthman

Tetrahedron 2012, 68, 7035–7040.

^{*} Equally contributing authors.

The Authors' Contribution to Papers I-IV

- I Contributed to the formulation of the research problem, performed half of the experimental work, contributed considerably to the interpretation of the results, and writing of the manuscript.
- **II** Contributed significantly to the formulation of the research problem, performed or supervised all experimental work, interpreted the results, and wrote the major part of the manuscript.
- **III** Formulated the research problem, performed the major part of the experimental work and interpretation of the results, wrote the manuscript.
- **IV** Formulated the research problem, performed or supervised all experimental work, interpreted the results, and wrote the manuscript.

List of Abbreviations

ADPAdenosine diphosphateADPrAdenosine diphosphate riboseAsnAsparagineAspAspartic acidArArylaqAqueousBoctert-ButyloxycarbonylBnBenzylBuButylt-BuyCDICDICarbonyldimidazoleCRCalorie restrictionDAPI4',6-Diamidino-2-phenylindoleDDQ2,3-Dichloro-5,6-dicyano-1,4-benzoquinoneDFTDisobutyl aluminium hydride
ADPrAdenosine diphosphate riboseAsnAsparagineAspAspartic acidArArylaqAqueousBoctert-ButyloxycarbonylBnBenzylBuButylt-Butert-ButylCDICarbonyldiimidazoleCRCalorie restrictionDAPI4',6-Diamidino-2-phenylindoleDDQ2,3-Dichloro-5,6-dicyano-1,4-benzoquinoneDFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
AsnAsparagineAspAspartic acidArArylaqAqueousBoctert-ButyloxycarbonylBnBenzylBuButylt-BuEutylCDICarbonyldiimidazoleCRCalorie restrictionDAPI4',6-Diamidino-2-phenylindoleDDQ2,3-Dichloro-5,6-dicyano-1,4-benzoquinoneDFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
AspAspartic acidArArylaqAqueousBoctert-ButyloxycarbonylBnBenzylBuButylt-Butert-ButylCDICarbonyldiimidazoleCRCalorie restrictionDAPI4',6-Diamidino-2-phenylindoleDFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
ArArylaqAqueousBoctert-ButyloxycarbonylBnBenzylBuButylt-Butert-ButylCDICarbonyldiimidazoleCRCalorie restrictionDAPI4',6-Diamidino-2-phenylindoleDDQ2,3-Dichloro-5,6-dicyano-1,4-benzoquinoneDFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
aqAqueousBoctert-ButyloxycarbonylBnBenzylBuButylt-Butert-ButylCDICarbonyldiimidazoleCRCalorie restrictionDAPI4',6-Diamidino-2-phenylindoleDDQ2,3-Dichloro-5,6-dicyano-1,4-benzoquinoneDFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
Boctert-ButyloxycarbonylBnBenzylBuButylt-Butert-ButylCDICarbonyldiimidazoleCRCalorie restrictionDAPI4',6-Diamidino-2-phenylindoleDDQ2,3-Dichloro-5,6-dicyano-1,4-benzoquinoneDFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
BnBenzylBuButylt-Butert-ButylCDICarbonyldiimidazoleCRCalorie restrictionDAPI4',6-Diamidino-2-phenylindoleDDQ2,3-Dichloro-5,6-dicyano-1,4-benzoquinoneDFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
BuButylt-Butert-ButylCDICarbonyldiimidazoleCRCalorie restrictionDAPI4',6-Diamidino-2-phenylindoleDDQ2,3-Dichloro-5,6-dicyano-1,4-benzoquinoneDFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
t-Butert-ButylCDICarbonyldiimidazoleCRCalorie restrictionDAPI4',6-Diamidino-2-phenylindoleDDQ2,3-Dichloro-5,6-dicyano-1,4-benzoquinoneDFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
CDICarbonyldiimidazoleCRCalorie restrictionDAPI4',6-Diamidino-2-phenylindoleDDQ2,3-Dichloro-5,6-dicyano-1,4-benzoquinoneDFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
CRCalorie restrictionDAPI4',6-Diamidino-2-phenylindoleDDQ2,3-Dichloro-5,6-dicyano-1,4-benzoquinoneDFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
DAPI4',6-Diamidino-2-phenylindoleDDQ2,3-Dichloro-5,6-dicyano-1,4-benzoquinoneDFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
DDQ2,3-Dichloro-5,6-dicyano-1,4-benzoquinoneDFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
DFTDensity functional theoryDIBAL-HDiisobutyl aluminium hydride
DIBAL-H Diisobutyl aluminium hydride
DIPA Diisopropylamine
DMAP 4-Dimethylaminopyridine
DMF Dimethylformamide
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
d.r. Diastereomeric ratio
equiv Equivalent(s)
Et Ethyl
FITC Fluorescein isothiocyanate
FOXO Forkhead box protein O
GBM Gliomablastoma
Gln Glutamine
Glu Glutamic acid
h Hours
HD Huntington's disease
HDAC Histone deacetylase
His Histidine
HPLC High performance liquid chromatography
HRMS High resolution mass spectrometry
IC ₅₀ Inhibitor concentration required to inhibit an enzyme by 50%
Ile Isoleucine
Inh Inhibitor

<i>i</i> -Pr	Isopropyl
IR	Infrared
LC	Liquid chromatography
LED	Light emitting diode
Leu	Leucine
Lys	Lysine
Me	Methyl
min	Minutes
Ms	Mesyl
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Microwave
NAD ⁺	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
n.d.	Not determined
NMR	Nuclear magnetic resonance
NSC	Neural stem cells
PAL	Photoaffinity labeling
PDB ID	Protein data bank identity
PFA	Paraformaldehyde
Ph	Phenyl
Phe	Phenylalanine
ppm	Parts per million
Pro	Proline
PSA	Polar surface area
<i>p</i> -TSA	Toluenesulfonic acid
ру	Pyridine
RNA	Ribonucleic acid
rt	Room temperature
SAR	Structure-activity relationship
SIRT	Silent information regulator type
SD	Standard deviation
TBAA	Tertabutylammonium acetate
TBAF	Tetrabutylammonium fluoride
TBDMS	tert-Butyldimethylsilyl
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TPAP	Tetrapropylammonium perruthenate
UV	Ultraviolet
VCD	Vibrational circular dichroism

Table of Content

1	INTRODUCTION	1			
	1.1 SIRTUINS	1			
	1.2 THE HUMAN SIRTUINS	2			
	1.2.1 Enzymatic activities of the SIRTs	2			
	1.2.2 Mechanism of the lysine deacetylation reaction				
	1.2.3 Structure of the sirtuins				
	1.2.4 Aspects on the biological function of the sirtuins				
	1.2.5 Sirtuin modulators	8			
2	AIMS OF THE THESIS	11			
3	CHROMAN-4-ONES AND CHROMONES AS SIRT2 INHIBITORS (PAPER I AND II)				
	3.1 CHROMAN-4-ONES AND CHROMONES	13			
	3.2 CHROMAN-4-ONES AND CHROMONES AS SIRT2 SELECTIVE INHIBITORS (PAPER I)	14			
	3.2.1 8-Bromo-6-chloro-2-pentylchroman-4-one, a selective SIRT2 inhibitor	14			
	3.2.2 Synthesis of chroman-4-one and chromone derivatives based on lead compound 24				
	3.2.3 Biological evaluation and structure-activity relationship study				
	3.3 SECOND GENERATION CHROMAN-4-ONE BASED SIRT2 INHIBITORS				
	(PAPER II)	20			
	3.3.1 Synthesis of chroman-4-ones carrying heterofunctionalized alkyl groups in the 2-position	21			
	3.3.2 Evaluation of the SIRT2 inhibitory effect of trisubstituted chroman-4 ones and	07			
	Tetrasubstituted chromones	// 29			
	3.3.4 Structure-activity relationships.				
	3.3.5 Evaluation of antiproliferative properties of chroman-4-one based SIRT2 inhibitors				
	3.3.6 Studies of the effect of SIRT2 inhibition on brain tumor cells	35			
	3.4 SUMMARY OF PAPER I AND II	37			
4	IDENTIFICATION OF THE BINDING SITE OF THE CHROMAN-4-ONE BASED SIRT2 INHIBITORS (PAPER III)	39			
	4.1 Photoaffinity Labeling	39			
	4.1.1 Photoreactive groups	39			
	4.1.2 Identification of the photolabeled amino acid residues				
	4.2 IDENTIFICATION OF THE BINDING SITE OF THE CHROMAN-4-ONE BASED				
	SIRT2 INHIBITORS USING PHOTOAFFINITY LABELING	43			
	4.2.1 Design and Synthesis of PAL Probes for SIRT2 based on chroman-4-one Scaffold				
	4.2.2 Investigation of the SIRT2 inhibition of the PAL probes				
	4.2.3 Investigation of the photochemical properties of diazirine 107	45			

	4.2.4 Photoaffinity labeling of SIRT2		
	4.2.5 Summary of Paper III		
5	5 A SCAFFOLD REPLACEMENT APPRO	DACH TOWARDS NEW SIRT	
	INHIBITORS (PAPER IV)		
	5.1 Scaffold		
	5.1.1 Quinolones and cyclic sulfonamides		
	5.2 Synthesis of scaffold analogs c	F THE CHROMAN-4-ONES	
	5.2.1 Synthesis of trisubstituted quinolin-4-(1H)-a	me analogs55	
	5.2.2 Synthesis of derivatives based on the benzoth	iadiazine-1,1-dioxide scaffold56	
	5.2.3 Synthesis of saccharine derivatives		
	5.3 BIOLOGICAL EVALUATION AND STRU	CTURE-ACTIVITY RELATIONSHIP	
	STUDY OF THE NEW SCAFFOLD ANAL	DGS	
	5.3.1 Evaluation of the inhibitory activity towards	SIRT1-3	
	5.3.2 Structure-activity relationship study		
	5.3.3 Summary of Paper IV		
6	6 CONCLUDING REMARKS AND FUTU	URE PERSPECTIVES63	
7	7 ACKNOWLEDGEMENTS		
8	8 REFERENCES		

1.1 SIRTUINS

The sirtuin family of enzymes is conserved from bacteria to mammals.¹ The gene of the founding member of the family, <u>silent information regulator type 2 (sir2)</u>, was discovered in *Saccharomyces cerevisiae* about three decades ago.^{2,3} It was found to be involved in transcriptional repression. Later, it was discovered that the transcriptional silencing was mediated via nicotinamide adenine dinucleotide (NAD⁺) dependent deacetylation of N^{ε} -acetylated lysine residues of histones. Histones are polar proteins in the cell nucleus that pack and order DNA by acting as a kind of spool around which the DNA winds. The acetylation/deacetylation state of lysine residues within the protruding amino-termini from the histones regulates the affinity of histones for DNA. Deacetylation provides positively charged lysine residues producing a tight DNA-histone complex leading to gene silencing. The reverse reaction, lysine acetylation catalyzed by histone acetylation are two of several important posttranslational modifications; other examples are phosphorylation or methylation which can occur on histones and non-histone proteins.⁴⁻⁷

The sir2-like enzymes (sirtuins) constitute the class III of histone deacetylases (HDACs). They are distinct from the other classical HDAC classes (I, II and IV) by their unique requirement of NAD⁺ as co-substrate.^{8,9}

Extensive studies of the sirtuins were initiated in the beginning of 2000 when Guarente and co-workers discovered that sir2 extends the lifespan of yeast and lower organisms like worms.^{10,11} It was also suggested that sir2-like enzymes could be potential mediators of the life prolonging effects observed on calorie restriction (CR), a dietary regime of reduced calorie intake.^{12,13} Thus, it was suggested that also the mammalian homologs of sir2 are associated with aging and might act as mediators for a prolonged lifespan and health beneficial effect in higher organism kept on CR.¹⁴⁻¹⁹ This resulted in extensive research efforts on sirtuins aiming to understand the underlying mechanism of the observed SIRTmediated effects and subsequently also to the development of modulators of the sirtuins. Since then, other studies have been questioning the life prolonging effect of sir2-like enzymes It is still a controversial question whether sirtuins are mediating the longevity related to CR or not.^{20,21}

1.2 THE HUMAN SIRTUINS

The human family of sirtuins comprises seven different members (SIRT1–7)[†] with different cellular locations.^{1,22} SIRT1, 6 and 7 are mainly nuclear, SIRT2 is predominantly found in the cytoplasm and SIRT3–5 are localized in the mitochondria.²³

1.2.1 Enzymatic activities of the SIRTs

The enzyme reactions that are catalyzed by SIRTs are depicted in Figure 1. The main physiologically relevant reaction is the deacetylation of *N*-acetylated lysine residues (**A**).^{24,25} SIRT1–3 are efficient deacetylases; however, this activity is less pronounced for SIRT5–7 and remained undiscovered for a long time for SIRT4. Recently however, Rauh *et al.* screened 6800 human lysine acetylation sites for deacetylation by sirtuins and identified several new substrates for SIRT including substrates for SIRT4.²⁶ Other post-translational deacylation reactions have been reported for SIRT5 including demalonylation, desuccinylation and deglutarylation (**B**);^{27,28} whereas SIRT6 catalyzes the removal of long fatty acid acyl groups (**C**).²⁹ Also mono-ADP ribosyl (ADPr) transfer is reported to be catalyzed by SIRT4 and SIRT6 (**D**).^{30,31}



Figure 1. Enzymatic reactions catalyzed by SIRTs. In addition to the deacetylation of lysine residues (A), lysine demalonylation, desuccinylation and deglutarylation (B), the removal of fatty acyl groups (C) and mono-ADP-ribosylation (D) have been reported. The members of the sirtuin family catalyzing the various reactions are given on the arrows.

The primary function of the sirtuins is the deacetylation of lysine residues. Preliminary studies of the other SIRT-catalyzed reactions indicate that they also might be physiologically relevant. Protein targets have been identified and will be described briefly in section 1.2.4.

[†] The human sirtuins will be abbreviated SIRT in capital letters, the number indicates the isoform. The yeast sirtuin sir2 is written in small letters.

1.2.2 Mechanism of the lysine deacetylation reaction

The first step of the SIRT catalyzed deacetylation is a bond forming reaction between the carbonyl oxygen of the acetyl group and the anomeric C1-carbon of the ribose unit in NAD⁺ under release of nicotinamide (NAM). This affords the ADPr-peptidyl imidate intermediate I (Scheme 1).³²⁻³⁴ The precise mechanism of this step is still under discussion; however a highly dissociative asynchronous S_N2 type mechanism has been proposed to be most likely.^{32,35-37} The reaction sequence continues with a nucleophilic attack of the ribose 2'-OH group activated by a conserved histidine residue onto the imidate (I) generating a bicyclic intermediate.^{35,38} The subsequent elimination of the deacetylated protein forms a cyclic oxonium species which is captured by an approaching water molecule. A tetrahedral intermediate is formed which affords the final product, 2'-O-acetyl-ADP-ribose. The 2'-Oacetyl-ADP-ribose is in equilibrium with its 3'-O-acetyl isomer via a transesterfication reaction.³⁵



Scheme 1. Proposed reactions steps of the deacetylation reaction catalyzed by SIRTs.³²

After formation of the ADPr-peptidyl-imidate (I), there are two possibilities for the reaction to proceed; the above mentioned attack of the 2'-OH group eventually leading to deacetylation of the peptide substrate, or a nucleophilic attack of the released NAM on the anomeric carbon of the ribose. This latter reaction, known as base exchange leads to the reversal of the first step and reformation of NAD⁺ and the acetylated lysine substrate; considering NAM as a physiological inhibitor of the sirtuins.^{32,39}

1.2.3 Structure of the sirtuins

The seven human sirtuins vary in length and in amino acid sequence from SIRT1 being the largest isoform with 747 amino acids to the other considerably shorter sirtuins with 310 to 400 amino acids (Figure 2).⁴⁰ The enzymes share a common catalytic core of

approximately 260 amino acids which is highly conserved throughout the family.¹ This core is flanked by *N*- and *C*-terminal extensions with varying lengths as illustrated in Figure 2. The function of these extensions is not yet fully understood. However, they are proposed to be targets for post-translational modifications e.g. phosphorylation, methylation and ubiquitination, which presumably regulate the function and localization of the SIRTs.^{41,42}



Figure 2. The sirtuins consist of a conserved catalytic core of about 260 amino acids which is flanked by more diverse *N*- and *C*-terminal domains.

The conserved enzymatic core consists of a small and a large domain (Figure 3, **A**).^{43,44} The small domain contains a zinc binding module with strictly conserved cysteine residues for zinc binding and, an α -helical module. The bound Zn²⁺ ion has no direct catalytic function in the sirtuins, however it is required to maintain sirtuin activity; as replacement of the conserved cysteines with alanines in sir2 abolish the deacetylation activity.⁴⁴ The small domain has most variation in the amino acid sequence and the structurally most diverse part of the catalytic core region.⁴⁵

The large domain has a classical Rossmann fold motif typical for NAD⁺ binding proteins.⁴⁶ A central β -sheet formed by six parallel β -strands is surrounded by a variable number of α -helices depending on the isoform.^{43,45} Both domains are connected by four loops forming a cleft between the domains which is considered as the active site.^{44,45} The acetylated protein binds to the outside of the enzyme inserting the acetylated lysine side chain via a conserved hydrophobic tunnel in the cleft towards the active site.⁴⁷ NAD⁺ enters from the opposite side with the nicotinamide-ribose moiety pointing towards the center of the active site.^{44,48} The NAD⁺ binding site within the Rossmann fold can be divided into three sub-pockets; the A-site which accommodates the adenine moiety and the attached ribose group, the B-pocket which is binding the second ribose moiety and the C-pocket, which forms the NAM binding site (Figure 3, **B**).^{44,49}

Binding of the two substrates induces conformational changes within the enzyme. The peptide binding induces a shift of the small domain bringing both domains closer together. This shift positions conserved amino acid residues within the active site in an optimal arrangement to allow interactions with the substrates.^{34,47} Upon NAD⁺ binding the flexible co-factor binding loop containing several conserved amino acids gets ordered and approaches NAD⁺, forming hydrogen bonding interactions with the ribose moiety.^{44,50} As observed in several x-ray structures NAD⁺ binds in the presence of the acetylated peptide in

a constrained, "productive" conformation placing the NAM moiety in the C-pocket.⁴⁹ Binding of the positively charged pyridine ring in the hydrophobic environment of the Cpocket is believed to facilitate the release of NAM. This extended conformation of NAD⁺ also positions the ribose in an optimal position for the nucleophilic attack of the acetyl group. Without a peptide substrate, NAD⁺ binds in other so called "nonproductive" conformations that are not compatible with an enzymatic reaction.^{44,49}



Figure 3. (**A**) Crystal structure of apo-SIRT2 for the illustration of the overall structure of the SIRTs.⁴³ (**B**) Illustration of the sub-pockets of the NAD⁺ binding site with NAD⁺ bound in a productive conformation in sir2.⁴⁹

1.2.4 Aspects on the biological function of the sirtuins

SIRTs have a broad spectrum of deacetylation targets ranging from histones to various transcription factors and other proteins like α -tubulin relating the enzymes to different cellular processes. These finding have proposed sirtuins as attractive targets for drug development efforts.^{25,51-53}

The focus of this thesis is the development of SIRT2 modulators. Below the various human sirtuins except SIRT2 will be discussed, as SIRT2 will be covered in more detail in section 1.2.4.2.

1.2.4.1 SIRT1 and SIRT3-7

SIRT1 is the best-studied member of the sirtuins due to the striking discovery that activation of sir2, its yeast homolog, leads to life extension in yeast. A multitude of protein substrates have been identified for SIRT1-mediated deacetylation including histones (H1, H3, H4) and transcription factors (p53, FOXO, NF- κ B) relating the enzyme to transcriptional silencing, metabolism, cell proliferation, apoptosis, insulin signaling, oxidative stress

responses, and neurodegeneration.^{25,51} The involvement in these different cellular processes has linked SIRT1 to various diseases such as type 2 diabetes, cancer, neurodegeneration, inflammation, and cardiovascular diseases.^{53,54}

The mitochondrial sirtuins, SIRT3–5 are mainly related to regulation of metabolism and oxidative stress responses.⁵³ SIRT3 is the best characterized enzyme of the three isoforms and was found to have impact on the acetylation state of e.g. acetyl CoA synthetase and regulation of the ATP levels in the cell.⁵⁴ In addition, SIRT3 has a function in the protection from oxidative stress-induced damage.^{53,55} Less is known regarding the SIRT4 and SIRT5 activity. It was found that SIRT4-mediated ADP ribosyl transfer activity is involved in insulin secretion and triggers also cell cycle arrest and DNA repair upon DNA damage.^{30,51,56} The latter study suggests that SIRT4 might act as tumor suppressor. SIRT5 activity is associated with ammonia disposal during fasting by activation of carbamoyl phosphate synthetase (CPS) 1 via desuccinylation.^{27,57}

SIRT6 is associated with genome stability through DNA repair and SIRT6 deficiency in mouse models leads to genomic instability, defective DNA repair, age-related phenotypes and premature deaths.⁵⁸ In addition, a frequent loss of SIRT6 was observed in tumors which points towards a role of SIRT6 in tumor suppression.⁵⁹ The deacetylation of histones H3K9Ac/H3K56Ac and mono-ADP-ribosylation of poly-ADPr polymerase (PARP) 1 might both contribute to this function.⁶⁰ In addition, the unique SIRT6-mediated removal of fatty acyl groups from lysines occurring on tumor necrosis factor α (TNF α), modulates its secretion.²⁹

SIRT7, is the least studied isoform and its biological function is poorly understood. It was found to deacetylate H3K18Ac and promote transcriptional silencing of genes of tumor suppressors and of proteins mis-regulated in cancer.^{59,61}

Although the post-translational modifications other than deacetylation catalyzed by SIRT4-6 might have a physiological relevance in living organisms, and potential protein targets have been identified, still more detailed studies are needed to fully understand their significance.

1.2.4.2 SIRT2

SIRT2 is predominantly located in the cytoplasm⁶² but is shuttled into the nucleus during mitosis.^{42,63} Increased SIRT2 levels are observed in the G_2/M phase and overexpression of SIRT2 results in a prolonged mitotic phase. These findings point towards a role of SIRT2 in a mitotic checkpoint⁶³ which ensures that cells exposed to any stress signal or containing damaged DNA will not be processed through mitosis.⁶⁴

SIRT2 has been reported to deacetylate histone H4 and other non-histone substrates such as α -tubulin, FOXO1, and p65.^{62,65,66} These substrates implicates that SIRT2 is involved in the regulation of the cell cycle, cell proliferation, apoptosis and metabolism.⁶⁴ SIRT2 has evolved as a potential therapeutic target for age-related diseases such as cancer and neurodegeneration.^{66,67}

SIRT2 in cancer

Regarding the role of SIRT2 in oncogenesis contradictory reports are found in the literature, it seems to act both as a tumor suppressor and a promoter.⁵⁹ For example, studies by Hiratsuka *et al.* showed that SIRT2 expression is suppressed in glioma cells.⁶⁸ SIRT2 as a tumor suppressor is also corroborated by the finding that SIRT2 knockout mice develop tumors in several organs (e.g. breast, liver, lung, pancreas) showing genetic instability and abnormal mitosis.⁶⁹ In addition, Kim *et al.* also found decreased SIRT2 expression levels in several human cancers such as glioma, breast, liver, and prostate cancers. The tumor suppressor function of SIRT2 might be associated with its regulation of mitosis proteins which ensure chromosomal stability⁶⁹ as well as deacetylation of H4K16 and α -tubulin.

On the other hand, Ying and co-workers discovered that SIRT2 is required for survival of C6 glioma cells⁷⁰ and overexpression contributed to tumor cell growth in liver tissue; suggesting oncogenic properties of SIRT2.⁷¹ Lui *et al.* reported a SIRT2-stabilizing effect of the Myc-oncoprotein.⁷² This oncoprotein is frequently overexpressed in several cancers (e.g. pancreatic tumors and neuroblastoma) promoting cancer cell proliferation, whereas inhibition/knockdown of SIRT2 counteract the proliferation.⁷² In line with these results, down-regulation of SIRT2 has shown to reduce the proliferation in HeLa cells,⁷³ as well as in liver⁷¹ and pancreatic carcinomas.⁷² Furthermore, inhibition of SIRT2 by the selective inhibitor AGK-2 (**6**, Figure 4) has been shown to induce apoptosis in C6 glioma cells.^{70,74} AEM2, (**7**, Figure 4) another small-molecule SIRT2 inhibitor has been shown to reduce cancer proliferation via the a decrease in p53 deacetylation in non-small-cell lung cancer cells (A549 and H1299).⁷⁵

SIRT2 and neurodegeneration

In general, a neurotoxic effect is associated with the enzymatic activity of SIRT2.^{66,67} It has been shown that SIRT2 is abundant in the brain and it accumulates in the central nervous system with aging.⁷⁶ A study showed that inhibition by AGK-2 (**6**, Figure 4) and SIRT2 knockouts rescued neuronal cells from α -synuclein-mediated toxicity in a Parkinson's disease (PD) model.^{77,78} The neuroprotective effect is caused by formation of fewer and larger α -synuclein inclusions. The molecular mechanism underlying a SIRT2-mediated inhibitory effect on α -synuclein aggregation is not yet fully understood. However, the inhibition of α -tubulin deacetylation has been proposed as a link.^{67,79}

In another study, AGK-2 (6) and its structural analog AK-7 (10, Figure 4) have been shown to counteract progression of Huntington's disease (HD) by a decrease in cholesterol levels in neuronal cells via regulation of sterol biosynthesis.^{80,81} Recently, it was reported that treatment with 10 leads to reduced aggregation of mutant huntingtin and improved neuronal health in HD mouse models.⁸² However, the involvement of SIRT2 in HD is still under discussion.⁸³

1.2.5 Sirtuin modulators

The implication of the SIRTs in numerous cellular processes and their putative involvement in disease states such as cancer, neurodegeneration, inflammation, cardiovascular diseases or diabetes⁵³ has led to a grown interest in the development of SIRT modulators. As mentioned above the involvement in biological processes is complex and either inhibition or activation of a specific isoform is required.

1.2.5.1 Inhibitors

A series of SIRT inhibitors is shown in Figure 4. NAM (1) is an endogenous inhibitor of all members of the sirtuin enzyme family, and it is released during the enzymatic reaction. The first synthetic SIRT inhibitor, sirtinol (2), was discovered in 2001 in a high-throughput screening (HTS) campaign which aimed for potential sir2 inhibitors. It was found to inhibit sir2 and SIRT2.⁸⁴ Numerous other screening campaigns were initiated mainly focusing on SIRT1 and SIRT2 leading to the discovery of a variety of different compounds;⁸⁵ e.g. a thiobarbiturate-substituted 2-hydroxynaphthyl based SIRT1/2 inhibitor called cambinol (3).⁸⁶



Figure 4. Small-molecular SIRT1/2/3 inhibitors.

SAR studies of cambinol were conducted and increased potency and selectivity for SIRT2 was obtained by N1-substitution with hydrophobic substituents (*n*-butyl, Bn).^{87,88} Recently, replacement of the thiobarbiturate-like heterocycle with a five-membered ring systems (4) yielded potent SIRT1-3 selective inhibitors. The isoform selectivity can be controlled by variation of the heteroatom X (=N or O) and R³- and R⁶-substituents; this led

to one of the most potent and selective SIRT3 inhibitors (4) (IC₅₀=6 μ M).⁸⁹ Suzuki *et al.* reported the highly potent SIRT2 inhibitor **5**.⁹⁰ The 2-anilinobenzamide core originates from a screen to identify SIRT1 inhibitors using a library including nicotinamide- and benzamide-like structures.⁹¹ Other potent SIRT2 inhibitors are the previously mentioned AGK-2 (**6**),⁷⁷ AEM2 (**7**),⁷⁵ and AK-7 (**10**).⁸¹ Other inhibitors identified in HTS are the kinase inhibitor **8**,⁹² the indole based SIRT1 inhibitor Ex-527 (**9**),⁹³ the tenovins (**11**)⁹⁴ and splitomicin-analogs like **12**.⁹⁵

The suggested binding site for the small-molecular inhibitors are the B- and C-pockets of the NAD⁺ binding site and/or the acetyl lysine binding channel located between the two domains.^{77,85,87,95} However, for most of the inhibitors the binding sites remain elusive.

Scientist have also taken advantage of the unique catalytic mechanism of the SIRTs and developed mechanism- and substrate-based inhibitors. Peptide analogs with a thioacetylated ε -amino group as mimic for the acetylated lysine residue of natural substrates such as of α -tubulin or p53 (13) have revealed highly potent inhibitors.⁹⁶ In addition, small peptide-mimicking derivatives resulted in highly potent pan-inhibitors of SIRT1–3 (14).⁹⁷



Figure 5. Example of mechanism-based inhibitors containing a thioacetylated lysine residue.

In the past, the development of SIRT inhibitors was limited to SIRT1-3. This was mainly due to the limited possibilities to determine the inhibitory activity against SIRT4-7 in the enzyme assays. Because they only show weak or no deacetylation activity to known protein substrates. However, the discovery of enzymatic activities other than deacetylation for SIRT5 and SIRT6 (see section 1.2.1) and the expanding scope of protein targets for SIRT4-7-mediated deacetylation enables now the development of inhibitors for these isoforms.

Maurer *et al.* identified the first small-molecular SIRT5 inhibitor after establishment of an *in vitro* assay using a Cbz-protected N^{ε} -succinylated lysine substrate carrying a *C*-terminal coumarin moiety allowing determination of the inhibitory activity through a fluorescent readout.⁹⁸ Screening of an in-house library including thiobarbiturate-based compounds like **3** identified a panel of potent analogs with IC₅₀ values between 2.3 and 39 μ M for SIRT5. Analog **15** showed the best selectivity for SIRT5 and forms a potential lead compound for further optimization.⁹⁸ A virtual database screen using a SIRT6 crystal structure as template, identified several compounds which significantly decreased SIRT6 deacetylation activity with **16** being the most potent and selective SIRT6 inhibitor.⁹⁹ Substrate-based approaches utilizing the characteristic enzymatic activities of SIRT5 and SIRT6 in desuccinylation and fatty acyl group removal, respectively, furnished potent selective mechanism-based SIRT5 (17) and non-selective SIRT6 (18) inhibitors.^{100,101}



Figure 6. Small-molecular and peptide-based inhibitors of SIRT5 and SIRT6.

1.2.5.2 Activators

As mentioned in previous sections, activation of sirtuins might provide beneficial health effects. Resveratrol (**19**, Figure 7), a natural occurring stilbenoid produced in several plants, was identified as a SIRT1 activator in a screening study.¹⁰² Also the naturally occurring flavonoids quercetin (**20**) and fisetin (**21**) and the chalcone butein (**22**) have been attributed to have a similar effect on SIRT1.¹⁰² Later, substituted 6-azaindoles, e.g. **23**, have been reported as more potent SIRT1 activators.¹⁰³ However, there is an ongoing debate whether or not the observed effect on SIRT1 activity is an artifact of the screening assay. It was shown that the enzyme activation is dependent on the fluorophore that is attached to the peptide substrate used in the test assay.¹⁰⁴



Figure 7. Putative SIRT1 activators.

Quercetin and fisetin are representatives of a class of oxygen-containing bicyclic ringsystems, called chromones. The chromone structure is highlighted in blue in the structure of **20** and **21** in Figure 7. For a long time our group has been interested in chromones and chroman-4-ones, as building blocks for the development of biologically active compounds, e.g. as scaffold for peptidomimetics and as kinase inhibitors.¹⁰⁵⁻¹⁰⁸ Therefore, we were also interested to investigate if functionalized chromones and chroman-4-ones could serve as SIRT modulators. The overall aim of the work presented in this thesis was the design, synthesis and biological evaluation of scaffold based sirtuin inhibitors.

The specific objectives of the thesis were:

- Synthesis of chroman-4-one and chromone derivatives as SIRT2 inhibitors and identification of features essential for activity in a structure-activity relationship study (Paper I).
- Improvement of physicochemical properties of the chroman-4-one and chromonebased SIRT2 inhibitors (Paper II).
- Identification of the binding site of the SIRT2 selective chroman-4-one based inhibitors using a photoaffinity labeling approach. (Paper III).
- Replacing the chroman-4-one/chromone scaffold with other heterofunctional bicyclic frameworks (Paper IV).

3.1 CHROMAN-4-ONES AND CHROMONES

Chromones and chroman-4-ones are oxygen-containing bicyclic frameworks (scaffolds) (Figure 8) found in numerous naturally occurring compounds.^{109,110} The most frequently found natural representatives are poly-hydroxylated and/or methyoxylated 2-arylchromones (flavonoids) which are found in leaves, tea, fruits, berries and olives.¹¹¹ The scaffolds have been classified as privileged structures^{112,113} since chroman-4-ones and chromones show different pharmacological effects depended on their substitution pattern, e.g. antiinflammatory, antibacterial, antiviral, or anticancer properties.^{110,114-118}



Figure 8. Chemical structure of the chromone and chroman-4-one scaffold.

A summary of common retrosynthetic routes to obtain substituted chromones and chroman-4-ones is illustrated in Figure 9. The most common approach towards 2-aryl chromones (route 1) involves the acid-catalyzed cyclization of 1,3-diketones afforded from a Baker-Venkataraman rearrangement of O-acylated 2'-hydroxyacetophenones.^{108,119,120} An alternative route consists of a transition-metal or organo catalyzed ring-closure reaction of O-alkynoylphenols (route 2).¹²¹⁻¹²³ 2-Alkyl chromones can be obtained from the corresponding chroman-4-ones via oxidation or 2,3-elimination reactions (route 3).105,124 2-Substituted chroman-4-ones can be synthesized via oxa-Michael cyclization of an α,β unsaturated intermediate formed from aldol condensations of 2-hydroxyacetophenones and aldehydes (route 4).105,125 Asymmetric oxa-Michael cyclization has also been reported.126,127 Chroman-4-ones can also be formed via a 1,4-conjugate addition onto chromones (R=H) (route 5).¹²⁸ Asymmetric methods for this approach has been developed to yield the 2-alkyl chroman-4-ones in good yields and high ee.129



Figure 9. Retrosynthetic outline of common synthetic methods to obtain substituted chromone and chroman-4-one derivatives.

3.2 CHROMAN-4-ONES AND CHROMONES AS SIRT2 SELECTIVE INHIBITORS (PAPER I)

3.2.1 8-Bromo-6-chloro-2-pentylchroman-4-one, a selective SIRT2 inhibitor

In an initial study, a small set of chromone and chroman-4-one based compounds were tested against human SIRT1-3 to see if the scaffolds could serve as frameworks for sirtuin modulators (data not shown). Interestingly, 8-bromo-6-chloro-2-pentylchroman-4-one **24** gave excellent inhibition (88%) of SIRT2 at 200 μ M concentration using a fluorescence-based assay (see Scheme 4). The compound also showed high selectivity for SIRT2 over SIRT1 and SIRT3. The IC₅₀ value of **24** was 4.3 μ M, thus **24** exhibited similar potency as previously reported SIRT inhibitors (Figure 4)



Figure 10. Structure of 8-bromo-6-chloro-2-pentylchroman-4-one, the first chroman-4-one based inhibitor.

The inhibitory activity of **24** was verified by two additional assays. First, analysis of the SIRT2-mediated deacetylation of acetylated α -tubulin was carried out. Decreased deacetylation of the substrate was observed as a result of SIRT2 inhibition (Figure 11, **A**). Secondly, a SIRT2 activity assay based on the release of radioactive ¹⁴C-nicotinamide was performed in the presence of an acetylated peptidic substrate (RSTGGK(Ac)APRKQ) lacking a fluorophore (Figure 1, **B**). In this assay **24** showed 66% inhibition. Taken together, **24** was able to inhibit the deacetylation of three different substrates and is therefore considered as a true inhibitor of SIRT2.



Figure 11. Inhibition of SIRT2 mediated deacetylation reactions by compound 24. (A) Western blot analysis of the inhibition of SIRT2 mediated α -tubulin deacetylation by 24. The concentration of 24 was 200 μ M, measurements were done at 30 min and 1 h. (B) Inhibition by 24 of the SIRT2 mediated deacetylation of the acetylated peptide RSTGGK(Ac)APRKQ. The reaction was detected by formation of the reaction product ¹⁴C-nicotinamide.

Based on these encouraging results we wanted to explore the structure-activity relationship (SAR) around the lead compound. Thus, a series of analogs with alterations of the carbonyl group, replacement of the pentyl side chain with other substituents, as well as modifications of the substitution pattern of the aromatic ring was synthesized.

3.2.2 Synthesis of chroman-4-one and chromone derivatives based on lead compound 24

A series of chroman-4-one derivatives were synthesized according to a methodology reported previously by our group; a base-promoted aldol condensation of substituted 2'-hydroxyacetophenones and appropriate aldehydes followed by an intramolecular *oxa*-Michael addition (Scheme 2).¹⁰⁵ The alkyl substituent in the 2-position is defined by the nature of the applied aldehyde. The substituents on the aromatic part of the scaffold are determined by the substitution pattern of the 2'-hydroxyacetophenone. Different commercially available 3-, and/or 5-substituted 2'-hydroxyacetophenones were used in the synthesis. The reactions were conducted by heating ethanolic mixtures to 160–170 °C using microwave (MW) irradiation for 1 h in the presence of diisopropyl amine (DIPA) as base. The outcome of the reaction was strongly dependent on the substitution pattern of the used 2'-hydroxyacetophenones, with yields varying between 17% and 88%. In general, electron deficient 2-alkyl-chroman-4-ones can be synthesized in high yields, whereas use of electron-rich acetophenones as starting material resulted in an increased formation of by-products originating from the competing self-condensation reaction of the aldehyde causing purification problems that lowered the obtained yields (**28** and **31**).





^bCommercially available.

Chromone analogs of **24** and derivatives with alterations of the carbonyl group were synthesized according to the methods outlined in Scheme 3. Flavone **41** was prepared via esterification of 3'-bromo-5'-chloro-2'-hydroxyacetophenone (**40**) with benzoyl chloride in pyridine, followed by the base-promoted Baker-Venkataraman rearrangement yielding a diketo intermediate that formed **41** upon acid-catalyzed cyclization.¹⁰⁶

Scheme 3. Synthesis of chromone analogs 41 and 43 and the manipulation of the carbonyl functionality of 24 towards 44-46.^{*a*}



^{*a*}Reagents and conditions: (a) i. Benzoyl chloride, py, rt, 2 h; ii. KOH, py, 50 °C, 4 h; iii. HCl, AcOH, reflux, 14 h; (b) Hexanal, DIPA, EtOH, MW, 170 °C, 1 h, (c) Py·Br₃, CH₂Cl₂, rt, 2.5 h, *cis/trans* ratio 80:20; (d) CaCO₃, DMF, MW, 100 °C, 20 min; (e) NaBH₄, MeOH/THF, 0 °C \rightarrow rt, 15 min, 95:5 d.r.; (f) Et₃SiH, BF₃·Et₂O, CH₂Cl₂, -78 °C \rightarrow rt, 19 h; (g) *p*-TSA, MgSO₄, toluene, 90 °C, 1.5 h. ^{*b*}Isolated yield over three steps.

The 2-pentylchromone **43** was obtained via α -monobromination of **24** with Py•Br₃ followed by a dehydrobromination of **42** with CaCO₃ in DMF in an overall yield of 75%. Reduction of the carbonyl group in **24** using NaBH₄ was performed to yield chroman-4-ol **44** in a diastereomeric ratio (d.r.) of 95:5 according to ¹H NMR spectroscopy. Treatment of **44** with Et₃SiH and BF₃·Et₂O furnished 8-bromo-6-chloro-2-pentylchromane **45** in 44% yield. Exposure of **44** to catalytic amounts of *para*-toluenesulfonic acid (*p*-TSA) yielded the dehydration product **46**.

To investigate the inhibitory activity of the individual enantiomers of 24, the enantiomers were separated by preparative high-performance liquid column chromatography (HPLC) using a chiral stationary phase. To determine the absolute stereochemistry of the separated enantiomers, x-ray crystallography was intended to be used. Unfortunately, all attempts to obtain suitable crystals failed. Alternatively, the absolute configuration of small chiral molecules can be determined by the comparison of experimental vibrational circular dichroism (VCD) spectra with predicted VCD spectra of the enantiomers. The VCD spectrum of a chiral molecule is the difference spectrum obtained by the absorption of leftand right circular polarized light in the infrared (IR) range. VCD spectra of low-energy conformations of the target structure can be predicted by density functional theory (DFT) calculations.¹³⁰ Highly flexible groups like the pentyl group in the 2-position of 24 result in many conformers which have to be considered in the DFT calculations. Therefore, the truncated ethyl-substituted analog was used for the DFT calculations as this change is not expected to alter the calculated VCD spectra.¹³¹ The predicted VCD spectra of the R- and Senantiomer were compared with the observed spectra from (+)-24 and (-)-24 (Figure 12). Comparison of the measured VCD spectrum of (-)-24 and the calculated spectrum of the S- enantiomer shows a good alignment of the bands in the frequency region between 1500 to 1100 cm⁻¹. Also the experimental data obtained for (+)-24 fitted equally well with the calculated spectrum of the R-enantiomer. On the basis of this, (-)-24 is likely to be the *S*-enantiomer and (+)-24 the R-enantiomer, respectively.



Figure 12. Comparison of the experimental VCD spectra of (-)-24 (blue) and (+)-24 (green) with the calculated spectra of the *S*-enantiomer (purple) and the *R*-enantiomer (red) of 24, respectively.

3.2.3 Biological evaluation and structure-activity relationship study

The synthesized derivatives were evaluated for their inhibitory activity for SIRT1–3 using an *in vitro* fluorescence-based assay (Scheme 4).^{132,133} The assay applies an acetylated fluorogenic peptide substrate resembling a short amino acid sequence of the natural SIRT-substrate p53. The assay consists of two steps; (i) deacetylation of the N^{ε} -acetylated lysine residue followed by (ii) addition of trypsin as a developer to release the fluorophore. The enzymatic activity is detected as a function of the measured fluorescence.

Scheme 4. Schematic illustration of the fluorescence-based assay used to determine the inhibitor activity of test compounds.



The results from the *in vitro* assay are summarized in Table 1. Several trisubstituted chroman-4-ones/chromones were potent inhibitors of the SIRT2 isoform (>70% inhibition at 200 μ M). They also showed high isoform selectivity over SIRT1 and SIRT3; in these tests the highest observed inhibition was 16% of SIRT3 by **36**.

The evaluation of the individual enantiomers of **24** revealed, that the *S*-stereoisomer was the more potent inhibitor with an IC₅₀ value of 1.5 μ M. The *R*-enantiomer had an IC₅₀ value of 4.5 μ M being equally potent as the racemic mixture (IC₅₀=4.3 μ M) and the oxidized chromone analog **43** (IC₅₀=5.5 μ M). The expected chair-like conformation of the tetrahydro-*4H*-pyran-4-one with the pentyl substituent in an equatorial position allows the two enantiomers to position the alkyl group in a similar way (Figure 13). Therefore, only minor variations in activity for the individual enantiomers and the chromone analog are observed.



Figure 13. The enantiomers of 24 with an equatorial positioned R²-subsitutuent can adopt a similar binding mode which results in the minor difference observed in inhibitory activity

Removal of the chloride and bromide (25) from the lead structure resulted in an inactive compound revealing the importance of the substituents in the aromatic ring to achieve inhibition. Introduction of the electron-withdrawing fluorine groups (26) in the 6- and 8-position increased the activity slightly to 30%. However, the 6,8-dimethyl substituted 28 had a significant increase in activity compared to the difluorinated compound. Altogether these results revealed that the size of the 6- and 8-substituents is important and that electron-withdrawing properties can further improve the inhibitory activity. Removal of either substituent in the 6- or 8-position (29 and 32) decreased the inhibitory effect towards SIRT2. The 6-chloro substituted 29 was slightly more active with 55% inhibition compared the 8-bromo analog 32 (28% inh.). This indicates a stronger contribution of the substituent in the 6-position to the inhibitory activity. Replacement of the Cl-group with an electron withdrawing nitro group (30) resulted in an equally active compound whereas an analog with an electron donating methoxy group (31) was less active exhibiting only 20% inhibition.

6-Bromochroman-4-one (**39**), which lacks a substituent in the 2-position did not inhibit SIRT2 at a 200 μ M concentration and revealed the important contribution of the 2-alkyl group to the inhibitory effect observed for the chroman-4-ones.

Table 1 Results from evaluation of compounds **24–39**, **41**, and 43–46 in a SIRT1–3 activity assay.^{*a*}

		R ⁶				R^2 R^6 C	R ²
	^{R⁻} 24-39	R*	41, 43	^{R-} 44	^{K*} 45	^{K*} 46	
Compd	R ²	R ⁶	\mathbb{R}^8	Inhibition SIRT1	n ± SD at 200 SIRT2) μM (%) ^b SIRT3	IC ₅₀ for SIRT2 (μ M) ^{c,d}
24	`\$\$	Cl	Br	6.2 ± 1.4	88 ± 0.9	2.6 ± 1.3	4.3 (3.5–5.4)
R -24	×\$\$~~~~~	Cl	Br	5.3 ± 3.1	70 ± 0.8	3.9 ± 1.2	4.5 (3.5–5.9)
S -24	×\$~~~~	Cl	Br	3.4 ± 5.4	91 ± 0.8	3.9 ± 2.4	1.5 (1.3–1.7)
25	`\$ ```````````````````````````````````	Н	Н	8.5 ± 1.0	4.9 ± 4.8	0.8 ± 2.6	n.d.
26	`\$ <u>\$</u>	F	F	0.4 ± 0.1	30 ± 1.3	9.6 ± 2.5	n.d.
27	`\$\$	Br	Br	0.6 ± 0.1	92 ± 1.2	6.5 ± 3.1	1.5 (1.3–1.7)
28	`***~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	CH ₃	CH_3	3.2 ± 3.2	83 ± 0.7	2.4 ± 3.1	6.2 (4.7–8.1)
29	×**	Cl	Н	2.7 ± 2.3	55 ± 2.4	6.0 ± 2.4	n.d.
30	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	NO_2	Н	4.7 ± 5.6	58 ± 0.7	16 ± 2.4	n.d.
31	×**	OCH ₃	Н	10.3 ± 1.8	20 ± 4.1	9.5 ± 3.0	n.d.
32	×\$~~~~	Н	Br	7.1 ± 1.6	28 ± 1.1	6.7 ± 3.6	n.d.
33	×≹∕∕	Cl	Br	6.4 ± 9.7	76 ± 1.8	7.0 ± 4.6	10.6 (9.0–12.5)
34		Cl	Br	7.5 ± 2.8	57 ± 2.5	8.3 ± 1.5	n.d.
35	`₹	Cl	Br	2.8 ± 1.4	52 ± 1.0	4.4 ± 0.4	n.d.
36	, second	Cl	Br	6.9 ± 3.4	81 ± 0.7	16 ± 0.9	6.8 (5.8–8.0)
37	- NH	Cl	Br	19 ± 1.7	53 ± 1.7	20 ± 1.0	n.d.
38	· čer v v v v v v v v v v v v v v v v v v v	Cl	Br	23 ± 5.6	27 ± 1.6	13 ± 2.6	n.d.
39	Н	Br	Н	n.d.	-1.5 ± 3.6	n.d.	n.d.
41	, we have a second seco	Cl	Br	3.1 ± 3.0	20 ± 1.4	12 ± 2.6	n.d.
43	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	Cl	Br	9.8 ± 2.8	82 ± 0.4	4.5 ± 1.6	5.5 (4.8–6.2)
44	`````	Cl	Br	-5.5 ± 2.4	31 ± 3.0	3.6 ± 5.7	n.d.
45	``````````````````````````````````````	Cl	Br	-5.2 ± 2.3	38 ± 1.3	1.2 ± 0.5	n.d.
46	××××××××××××××××××××××××××××××××××××××	Cl	Br	7.1 ± 0.1	38 ± 1.2	2.0 ± 7.3	n.d.

^{*a*}Compounds exhibiting over 70% inhibition for SIRT2 shaded in grey. ^{*b*}SD, standard deviation, (n=3). ^{*c*}IC₅₀ (95% confidence interval). IC₅₀-values were determined using the Fluor de Lys assay for compounds that showed over 70% inhibition of SIRT2 at 200 μ M cocentration. ^{*d*}n.d.=not determined.

Incorporation of either a *n*-propyl (**33**, 76% inh., $IC_{50}=10.6 \mu M$) or *n*-heptyl side chain (**34**, 57% inh.) in the 2-position furnished less active inhibitors. Thus, the pentyl side chain had an optimal length among the explored *n*-alkyl groups. The observation that the *iso*-propyl-substituted chroman-4-one **35** and 2-phenylchromone **41** exhibited a decreased inhibitory effect with 52% and 20% inhibition, respectively; indicating that branched groups are not tolerated in the vicinity of the scaffold. However, introduction of an ethylene spacer between the ring systems as in **36** furnished a potent inhibitor with an IC₅₀ value of 6.8 μ M. Replacement of the phenyl ring with the larger indole moiety (**37** and **38**) strongly affected the inhibitory activity negatively, indicating a space limitation in the binding site where the R²-substituent binds.

In general, reduction or removal of the carbonyl group (44–46) resulted in a significant decrease in SIRT2 inhibition. The chroman-4-ol 44 as well as unsubstituted analogs inhibited SIRT2 to less than 40%.

In summary, the SAR study surrounding lead compound **24** has identified key elements crucial to achieve inhibition of SIRT2 (Figure 14). The study revealed that an exceptionally limited variation is allowed. Modifications or removal of the carbonyl group or any of the substituents influence the inhibitory effect of the chroman-4-ones negatively.



Figure 14. Summary of the SAR study carried out in Paper I focusing on the evaluation of the pentyl side chain, carbonyl group and the substituents on the aromatic ring.

3.3 SECOND GENERATION CHROMAN-4-ONE BASED SIRT2 INHIBITORS (PAPER II)

A major drawback with the first series of chroman-4-one based SIRT2 inhibitors was associated with their high lipophilicity. They showed poor water solubility which limited their application in *in vitro* tests on cancer cells due to precipitation at relevant test concentration. Therefore, 2-substituted chroman-4-one derivatives with increased hydrophilicity compared to the previously evaluated compounds were envisioned to be synthesized.

The lipophilicity of the chroman-4-ones was intended to be reduced via the introduction of heterofunctional groups in the 2-position of the scaffold (Figure 15). The Cland Br-substituents were kept as these groups were found to have beneficial effects on the inhibitory activity. Acetophenones with this substitution pattern gave the corresponding chroman-4-ones in high yields and the Cl- and Br-groups allow regioselective functionalization of these positions via Pd-catalyzed coupling reactions at a later stage. We also planned to evaluate small heterofunctional groups in the 3-position by the synthesis of a small series of tetrasubstituted chromones in order to study this yet unexplored position.

For the synthesis of the compounds we intended to use the previously successfully applied method of reacting 2'-hydroxyacetophenones with the appropriate aldehydes. Commercially available alcohols were considered as useful precursors for the desired aldehydes. The tetrasubstituted chromone derivatives were planned to be obtained via functionalization of the 3-position of the corresponding chroman-4-ones followed by introduction of the double bond.



Figure 15. Condensed overview of the heterofunctional groups intended to be introduced in the 2-position.

3.3.1 Synthesis of chroman-4-ones carrying heterofunctionalized alkyl groups in the 2-position

3.3.1.1 Chroman-4-ones with hydroxyalkyl groups and polyethylene glycol side chains in the 2-position

The synthesis towards these functionalized derivatives is illustrated in Scheme 5. The desired aldehydes (50–52) were synthesized from the corresponding mono-protected diols (47–49) (Scheme 5). Compounds 48 and 49 were obtained via a mono-protection protocol reported by McDougal *et al.* employing TBDMSCl and NaH as base.¹³⁴ The free alcohol groups were oxidized by Swern or Dess-Martin oxidations to give aldehydes 50–52¹³⁵ which were further reacted with 40 in the base-promoted aldol reaction described earlier. The chroman-4-ones (53–55) were obtained in good yields and sufficient purity to be directly used in the deprotection step. Using tetrabutylammonium fluoride (TBAF) in the subsequent deprotection resulted in an unexpected ring-opening reaction yielding 56 and 57 (Scheme 5). A ring-opening of the chroman-4-ones leading to an α,β -unsaturated intermediate which is attacked by the nucleophilic terminal OH-group could give rise to these compounds. Instead, a microwave-assisted deprotection using Selectfluor[®] reported by Shah *et al.* gave 58–60 in varying yield (16-78%) over three steps.¹³⁶

Scheme 5. Synthesis of chroman-4-one derivatives **58–60** carrying a terminal hydroxyl group with varying length of the alkyl spacer.^{*a*, *b*}



min; iii. Et₃N, -78 °C \rightarrow rt, 15 min; or **49**, Dess-Martin periodinane, CH₂Cl₂, rt; (b) **40**, DIPA, EtOH, MW, 170 °C, 1–2 h; (c) TBAF, THF, rt, overnight; (d) Selectfluor®, MeOH, MW, 150 °C, 30 min. [#]Isolated yields over three steps. ^cCommercially available.

The synthesis towards polyethylene glycol substituted chroman-4-ones is illustrated in Scheme 6. Swern oxidation of the alcohols yielded the corresponding aldehydes **61** and **62**. The ordinary work-up procedure of the oxidation involving addition of water and EtOAc had to be changed to a non-aqueous work-up due to the high water solubility of the compounds. The chroman-4-one reaction using the standard conditions yielded **63** and **64** in 20% and 18% yield at its best, respectively.

Scheme 6. General procedure for the synthesis of polyethylene glycol substituted chroman-4-ones 63 and 64.^{*a,b*}



^{*a*}Reagents and conditions: (a) i. (COCl)₂, DMSO, THF, -78 °C, 30 min; ii. **61–62**, -78 °C, 30 min; iii. Et₃N, -78 °C \rightarrow rt, 15 min; (b) **40**, DIPA, EtOH, MW, 150 °C, 1 h. ^{*b*}Isolated yield over two steps.

Attempts to optimize the reaction with larger amounts of base (1.1–2 equiv), variation of reaction time (0.5–3 h) and temperatures (100–170 °C) did not increase the yields. In addition, several uncharacterized by-products were formed which complicated the purification process. The by-products could originate from polymerization under the basic conditions employed.¹³⁷ Derivative **64** could not be isolated in sufficient purity for biological testing.

3.3.1.2 Chroman-4-ones carrying an terminal ester and amide groups in the R²- side chain

Beside a terminal hydroxyl group, an ester moiety was desired in the R²-position. The synthetic procedure is outlined in Scheme 7 and was commenced by the synthesis of the aldehydes **65** and **66**. They were obtained from γ -butyrolacton and δ -valerolacton via a ring-opening reaction with MeOH and subsequently oxidized to yield **65** and **66**. These were reacted with **40** in the microwave reactor at 170 °C using piperidine or DIPA as base to furnish **67** and **68** in moderate yields (40–64%).

Scheme 7. Synthesis of ester derivatives of 2,6,8-trisubstituted chroman-4-ones.^a



^aReagents and conditions: (a) Et₃N, MeOH, rt, 18 h; (b) SO₃·py, Et₃N, DMSO, rt, 14 h; or i. (COCl)₂, DMSO, THF, −78 °C, 30 min; ii. Appropriate alcohol, -78 °C, 30 min; iii. Et₃N, −78 °C→rt, 15 min; (c) **40**, piperidine or DIPA, EtOH, MW, 170 °C, 0.5–1 h.

As esters are prone to hydrolysis at physiological pH an inevitable next step was to synthesize the corresponding carboxylic acid analogs. Simple hydrolysis of the ester moiety of **68** under basic conditions using LiOH or Me₃SnOH, was unsuccessful and resulted instead in the ring opened retro-Michael reaction product with partial ester hydrolysis. Instead, two consecutive oxidation steps of the hydroxyl group of **58** and **59** (Scheme 8) provided the carboxylic acids **69** and **70** in good yields. The carboxylic acid gave then access to hydrolytically more stable amide analogs. The amides were acquired in good to excellent yields via reaction of the CDI-activated carboxylic acids **69** and **70** with various amines (Scheme 8). Reaction of the acids with acetamide oxime gave the corresponding bioisosteric oxadiazoles (**76–77**) upon heating.

Scheme 8. Synthesis chroman-4-one based carboxylic acids 69–70, amide 71–75, and oxadiazoles 76–77.^{*a*}



^{*a*}Reagents and conditions: (a) Dess-Martin periodinane, CH₂Cl₂, rt, 0.75–1 h; (b) NaClO₂, NaH₂PO₄·2H₂O, amylene, H₂O, THF, 0 °C \rightarrow rt; (c) i. **69–70**, CDI, CH₂Cl₂/DMF, 0 °C, 30 min; ii. appropriate amine, 0 °C \rightarrow rt, 2–14 h; (d) i. **69–70**, CDI, MeCN/DMF, rt, 30 min; ii. Acetamide oxime, 85 °C, 14–19 h.

3.3.1.3 Introduction of heteroaromatic aryl groups and saturated heterocycles in the 2-position

In order to decrease the lipophilicity and to further explore the influence of the phenyl ring in **36**, pyridine rings and morpholine, piperidine and piperazine moieties were planned to be incorporated. Commercially available 3-pyridyl-1-propanols were oxidized via a Swern oxidation and the obtained aldehydes were reacted with **40** to yield the pyridyl-substituted **78–80** in moderate yields over two steps as outlined in Scheme 9. However, efforts to obtain substituted phenethyl derivatives were however unsuccessful.

Scheme 9. General synthetic procedures towards the pyridyl-substituted chroman-4-ones $78-80.^{a, b}$



^{*a*}Reagents and conditions: (a) i. (COCl)₂, DMSO, THF, -78 °C, 30 min; ii. Appropriate alcohol, -78 °C, 30 min; iii. Et₃N, -78 °C \rightarrow rt, 15 min; (b) **40**, DIPA, EtOH, MW, 170 °C, 1 h. ^{*b*}Isolated yields over two steps.

The initially applied strategy (Scheme 9) was also attempted for the introduction of the saturated heterofunctional groups. However, Swern oxidation of 3-morpholinopropan-1-ol or the use of other protocols (Dess-Martin periodinane, TPAP, TEMPO, CrO₃)^{135,138} did not afford the desired aldehyde **81** (Scheme 10) in significant amounts. The aldehyde could be instead prepared by a 1,4-conjugate addition of morpholine to acrolein (Scheme 10). Stirring morpholine with an excess of acrolein at room temperature overnight yielded **81** after co-evaporation of unreacted acrolein. With **81** in hand, the aldehyde was reacted with **40** using the standard protocol which however only gave small amounts of **82** according to NMR analysis. Attempts to optimize the yield did not improve the outcome of the reaction.

Scheme 10. Synthetic attempts towards chroman-4-ones 82.^a



^{*a*}Reagents and conditions: (a) MeCN, MgSO₄, rt, overnight, 90% crude yield; (b) **40**, DIPA, EtOH, 170 °C, 1 h; (c) i. (COCl)₂, DMSO, THF, -78 °C, 30 min; ii. alcohol, -78 °C, 30 min; iii. Et₃N, -78 °C \rightarrow rt, 15 min.
In a third approach, **82** was intended to be synthesized via a displacement of a terminal mesyl group using **84** as an intermediate (Scheme 10). Swern oxidation of 3-(*tert*-butyldimethylsilyl)-1-propanol afforded **83**. However, again the chroman-4-one formation was problematic yielding **84** in only trace amounts together with several uncharacterized byproducts.

Instead, the previously synthesized analog of **84** comprising one additional methylene group (**58**) (Scheme 5) was used. Mesylation of **58** (Scheme 11) using mesyl chloride and Et₃N in CH₂Cl₂ yielded **85** which was directly reacted with morpholine using microwave heating to produce **86** in 51% yield. Also piperidine and mono-Boc-protected piperazine were successfully applied in the synthesis. However, it was not possible to separate the Boc-protected piperazine derivative (**88**) from by-products using flash column chromatography, ion-exchange chromatography or acid-base-extraction, neither removal of the Boc-group facilitated the purification process. Therefore, only compounds **86** and **87** were evaluated for their biological activity.

Scheme 11. Synthesis of chroman-4-ones **86–89** with aliphatic heterocyclic rings in the 2-position.^{*a*}



^{*a*}Reagents and conditions: (a) MsCl, Et₃N, CH₂Cl₂, 0 °C \rightarrow rt; (b) amine, THF, MW, 120–150 °C, 1 h. (c) 3 M HCl, MeOH, rt, overnight. ^{*b*}Isolated yields over two steps. No pure product was obtained.

In addition to the above described monocyclic heterofunctionalities, also two different bicyclic ring systems were chosen to be investigated, i.e. quinolin-6-yl and 3,4-dihydro-2(1*H*)-quinolinone-6-yl (Scheme 12). 6-Bromo-3,4-dihydro-2(1*H*)-quinolinone (**90**) was prepared via an amide-forming reaction of 4-bromoaniline with β -ethoxyacryloyl chloride followed by acid-catalyzed intramolecular ring closure.¹³⁹⁻¹⁴¹ Reaction of **90** or commercially available **91** with acetal-protected acrolein in a ligand-free Heck reaction yielded **92a–b** (Scheme 12).¹⁴² Catalytic hydrogenation with 10% Pd/C and removal of the acetal group under acidic conditions produced **93a** in 34% over three steps (Scheme 12). Surprisingly, under the mild conditions (H₂-balloon, 10% Pd/C, room temperature) chosen for reduction of the double bond, in **92b** also the quinoline moiety was reduced to yield the corresponding 1,2,3,4-tetrahydroquinoline. When 1,4-cyclohexadiene and 10% Pd/C¹⁴³ were used instead no quinolone reduction occurred and after treatment with acid the desired aldehyde (**93b**) was obtained. The aldehydes were then reacted with **40** under standard conditions to yield **94a–b**.

Scheme 12. Synthesis of chroman-4-ones containing bicyclic heterofunctional groups in the side chain in the R^2 -position.^{*a*}



^{*a*}Reagents and conditions: (a) Acrolein diethyl acetal, Pd(OAc)₂, KCl, K₂CO₃, TBAA, DMF, 90 °C, overnight; (b) H₂, 10% Pd/C, MeOH, rt, 3 h or 1,4-cyclohexadiene, 10% Pd/C, EtOH, reflux, 4.5 h; (c) HCl (conc.), acetone, reflux, 2–4 h; (d) **40**, DIPA, EtOH, MW, 160–170 °C, 1.5–2 h. ^{*b*}Isolated yields over three steps.

3.3.1.4 Tetrasubstituted chromone derivatives with heterofunctionalities in the 3-position

The tetrasubstituted chromones **96–99** were synthesized as illustrated in Scheme 13. The mono-brominated chroman-4-one **95** was obtained by reaction of **36** with CuBr₂.¹⁰⁵ Treatment of **95** with NaN₃ in DMSO resulted in the formation of amine **96**,¹⁰⁵ which was acetylated with acetyl chloride in pyridine to form **97**. For the preparation of **98**, a SmI₂-mediated Reformatsky type reaction using tosyl cyanide as described earlier by Ankner *et al.* was used to introduce the nitrile moiety.¹²⁴ Subsequent oxidation with DDQ in dioxane furnished the desired target compound. Further, reduction of the nitrile group by means of DIBAL-H furnished enaminone **99** in 66% yield.

Scheme 13. Synthesis towards tetrasubstituted chromone derivatives.^a



^aReagents and conditions: (a) CuBr₂, CHCl₃:EtOAc, 2 h, reflux; (b) NaN₃, DMSO, 70 min, rt; (c) AcCl, pyridine, rt, overnight; (d) SmI₂, KHMDS, TsCN, THF, -78 °C→rt, *cis:trans* 29:71; (e) DDQ, dioxane, rt, 12 h; (f) DIBAL-H, CH₂Cl₂, -78 °C, 3 h. ^bIsolated over two steps.

3.3.2 Evaluation of the SIRT2 inhibitory effect of trisubstituted chroman-4 ones and tetrasubstituted chromones

The synthesized chroman-4-ones and chromones were evaluated for their inhibitory effect on the SIRT2 enzyme activity. The results are summarized in Table 2 and Table 3. The new series of 2-alkylsubsituted chroman-4-ones comprised highly potent SIRT2 inhibitors with retained selectivity over SIRT1 and SIRT3.

No.	R ²	Inhib (%) ^{<i>a,b</i>}	IC ₅₀ (μM) ^{c,d}	No.	R ²	Inhib (%) ^{a,b}	IC ₅₀ (μM) ^{c,d}
58	`€́ОН	18 ± 1.1	n.d.	74	° ↓ N H Ph	39 ± 0.9	n.d.
59	`{ ОН	52 ± 0.9	n.d.	75	N N	53 ± 1.4	n.d.
60	`€́OH	69 ± 0.5	n.d.	76	N O-N	77 ± 0.8	12.2 (10.2–14.7)
63	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	33 ± 2.0	n.d.	77	O-N N	49 ± 1.2	n.d.
67		80 ± 4.4	9.6 (6.8–13.6)	78	N.	74 ± 0.5	n.d.
68	, je o o o o o o o o o o o o o o o o o o	90 ± 0.5	2.0 (1.6–2.5)	79	`\$	86 ± 1.9	3.7 (3.1–4.5)
69	-≩∕∕́OH O	6.8 ± 1.5	n.d.	80	N N	73 ± 1.8	n.d.
70	,≹∕OH	7.6 ± 1.6	n.d.	86	N N	17 ± 10.7^{e}	n.d.
71	M O H N O	0.2 ± 4.8	n.d.	87	₹ N	40 ± 2.8^{e}	n.d.
72	° ↓ ↓ ↓ H	39 ± 0.9	n.d.	94a	N N N N N N N N N N N N N N N N N N N	59 ± 1.1	n.d.
73	State N H H H H H H H H H H H H H H H H H H	23 ± 1.8	n.d.	94b		56 ± 1.1	n.d.

Table 2. Results from evaluation of the less lipophilic 2-substituted 8-bromo-6-chlorochroman-4-ones in a SIRT2 inhibition assay.

^{*a*}SD, standard deviation (n=3). ^{*b*}Inhibition at 200 μ M inhibitor concentration. ^{*c*}IC₅₀ (95% confidence interval). IC₅₀ values were determined for compounds exhibiting >80% inhibition of SIRT2 at 200 μ M concentration or compounds evaluated in the cell proliferation assay. ^{*d*}n.d.=not determined. ^{*c*}The SIRTAinty assay was used for the determination.

The evaluated tetrasubstituted chromone-based derivatives comprised also selective SIRT2 inhibitors (Table 3). The acetamide **97** was the most potent chromone with 81% inhibition and an IC₅₀ value of 28.7 μ M.

No.	Structure	Inhibition (%) ^{a,b}	IC ₅₀ (μΜ) ^{ε,d}	
96	Cl NH ₂ Br	79 ± 1.5	n.d.	
97		81 ± 0.9	28.7 (21.4–38.5)	
98		50 ± 1.9	n.d.	
99		76 ± 1.9	n.d.	

Table 3. Results from evaluation of the tetrasubstituted chromones 96–99 in a SIRT2 inhibition assay.

^{*a*}SD, standard deviation (n=3). ^{*b*}Inhibition at 200 μ M inhibitor concentration. ⁴IC₅₀ (95% confidence interval). IC₅₀ value was determined for compounds exhibiting >80% inhibition. ^{*d*}n.d.=not determined.

The selectivity of the most potent inhibitors was further investigated by testing their inhibitory effect on members of other classes of HDACs. The test confirmed that the compounds exclusively inhibit the class III of lysine deacetylases (HDAC inhibition <10% at 200 μ M, data not shown).

In order to evaluate whether the introduction of the heterofunctional groups improved the physicochemical properties of the compounds like, properties like logP, logD and PSA were calculated for the new compounds. The results in Table 4, exemplified by the most potent inhibitors of the new series, clearly show more attractive physicochemical properties such as decreased clogP and clogD-values as well as a larger PSA.

the potent inhibitors 67–68, 76 and 79 from the new series.									
No.	IC ₅₀ (μΜ) ^c	MW	ACDlogP	ACDlogD pH 7.4	PSA (Ų)	HBD ^a	HBA ^b		
24	4.3	331.6	5.60	5.60	27.4	0	2		
36	6.8	365.7	5.57	5.57	27.4	0	2		
67	9.6	347.6	3.36	3.36	54.4	0	4		
68	2.0	361.6	3.77	3.77	54.4	0	4		
76	12.2	371.6	3.69	3.69	61.0	0	5		
79	3.7	366.6	4.19	4.18	37.6	0	3		

Table 4. Data of calculated physicochemical properties of lead compounds 24 and 36 and the potent inhibitors 67–68, 76 and 79 from the new series.

^aNumber of hydrogen bond donors. ^bNumber of hydrogen bond acceptors.

3.3.3 Putative binding site of the chroman-4-one based SIRT2 inhibitors

Molecular modeling is a powerful tool to visualize and understand the interaction of a bioactive compound with its biological target. Also within the SIRT field molecular modeling and docking studies have been widely used.

As mentioned in section 1.2.5.1 the binding site of many SIRT inhibitors remains unknown and only few substrate-based and small molecular inhibitors have been crystalized with the enzyme.144,145 However, molecular modeling has established the C-pocket and the nearby area as the presumable binding site for many potent small-molecular SIRT1/2 inhibitors such as AGK-2 (6),⁷⁷ cambinol (3)⁸⁷ and splitomicin analogs (12),⁹⁵ and Ex-527 $(9)^{144}$ (Figure 4). We therefore wanted to investigate whether this pocket could be a feasible binding site also for our compounds. Although there were crystal structures published of SIRT243,50,146 we decided to build a homology model.[‡] The reasons for this was that two of the available SIRT2 x-ray structures represent the apo-enzyme43,50 and their structures differ considerably compared to enzyme complexes with peptide substrate and/or NAD+present. Another SIRT2 structure in complex with ADP-ribose (ADPr) which binds to the NAD+ binding cleft was recently solved (PDB code 3ZGV), but it lacks an inhibitor.⁵⁰ The fourth structure (4L3O) is a complex of SIRT2 with a macrocyclic inhibitor (S2iL5) mimicking peptide substrate binding. This inhibitor differs considerably from the small molecular chroman-4-ones. Therefore, a homology model was constructed based on SIRT1 cocrystallized with NAD+ and an analog of Ex-527 (9, Figure 4) bound in the C-pocket (4I5I).¹⁴⁴ This structure has a high resolution and the inhibitor has a similar size and shape as the chroman-4-ones. A lysine residue from a Sir2-p53 peptide-NAD+-complex (2H4F)⁴⁸ was also included in the model as preliminary studies had shown that the chroman-4-ones do not act as peptide substrate competitive inhibitors (data not shown). The homology modeling was performed using the MOE software (v. 2012.10, Chemical Computing Group Inc.: Montreal). The 3-pyridylsubstituted inhibitor 79 was positioned in the C-pocket close to the location of the Ex-527 analog in the SIRT1 structure during the construction of the homology model.

In the final homology model, **79** fits nicely into the C-pocket of SIRT2 (Figure 16). The carbonyl oxygen of **79** interacts with a structural water molecule, which in turn interacts with a glutamine residue (Gln167) and NAD⁺. The chloride in the 6-position can form a halogen bonding interaction to the backbone carbonyl of the conserved His187 and the bromide in the 8-position is buried in a hydrophobic environment (Leu103, Phe119, Leu138, Phe190). The R²-substituent is located in a hydrophobic tunnel surrounded by Ile93, Pro94, Leu103, Leu138 accommodating NAM in the productive NAD⁺ binding mode. The pyridyl nitrogen interacts with Gln142 via hydrogen bonding.

A proposed mechanism for the chroman-4-one based inhibitors based on the results obtained from the molecular modeling could be, that they prevent NAD⁺ from binding in a productive or active conformation necessary for the deacetylation reaction to occur.

[‡] The molecular modeling was done by Dr. Marcus Malo.



Figure 16. (a) A schematic view of the interactions between the chroman-4-one-based inhibitor 79 and the human SIRT2 homology model. (b) The SIRT2 homology model with inhibitor 79 (magenta) present in the C-pocket indicating the same interaction points as in (a).

3.3.4 Structure-activity relationships

In this study we introduced 2-alkyl side chains substituted with heterofunctional groups and investigated the 3-position of the chromones containing different hydrogen bonding groups.

Replacement of the pentyl side chain in **24** (88% inh. IC₅₀=4.3 μ M) with a more polar ethylene glycol side chain (**63**) resulted in a significant decrease in activity (33% inhibition, Table 2). Introduction of a terminal hydroxyl group with C3–C5-carbon spacers (**58–60**) gave 18–69% inhibition; the activity increased with increasing linker length. However, **60** (C5-linker, 67% inhibition) is still less potent then the lead compound **24** (88% inhibition). This indicates that polar side chains and hydrogen bond donating groups are not favorable with a chain length up to five atoms. This is consistent with the molecular modeling results

(Figure 16) which indicate that the side chain in the 2-position is located in a rather hydrophobic channel.

Introduction of an hydrogen-bond accepting ester functionality (67 and 68) furnished potent inhibitors with 68 being one of the most potent inhibitors of the series with an IC_{50} value of 2.0 µM. Docking studies of 68 in our homology model revealed an hydrogen bonding interaction between the carbonyl oxygen and a glutamine residue (Gln142) (Figure 17). This hydrogen bond was also observed in the building process of the homology model with **79** (Figure 16). The methyl group of the ester is located in a small hydrophobic pocket. The corresponding carboxylic acids 69 and 70 were completely inactive presumable due to carboxylate formation at physiological pH. Negatively charged groups might be unfavorable in the lipophilic environment and narrow channel accommodating the R²-substituent. The replacement of the ester moiety in 67 (80% inh.) and 68 (90% inh.) with different amide functionalities including methyl, benzyl and *iso*-propyl amides (71-75) dramatically lowered the activity to less than 40%. Docking studies^{142,147} of **68** and **72** revealed positioning of the O- and N-methyl groups in a small hydrophobic pocket, rather than towards the aqueous solution (Figure 17). This orientates the single-bonded oxygen in the ester and the NHmoiety in the amide towards a hydrophobic site and makes the more polar amide unfavorable compared to the ester. This might explain the slightly increased activity of the more lipophilic dimethyl amide 75 with 53% inhibition. However, the narrowness of the tunnel turns the more sterically demanding dimethyl derivative into a less potent inhibitor compared to methyl ester 68.



Figure 17. The docked chroman-4-one analogs **68** (red) and **72** (green) in the SIRT2 homology model. The surface shows hydrophobic areas in green and hydrophilic regions in purple. The carbonyl oxygens are forming hydrogen bonds with Gln142 and the methyl groups are positioned in a small hydrophobic pocket. The polar *NH*-group on the amide is pointing towards a hydrophobic region. Only the water molecule interacting with the carbonyl group in the chroman-4-ones (W17) is shown.

The oxadiazole derivative with an ethylene linker (76, $IC_{50}=12.2 \mu M$) was equipotent to the corresponding methyl ester. Surprisingly, the bioisosteric replacement of the methyl ester moiety of 68 with an oxadiazole group (77) resulted in lower potency. Docking studies showed that 76 can adopt a similar binding pose as the ester whereas the methyl group of the longer oxadiazole 77 did not fit in the binding channel.

Replacement of the phenyl ring in **36** (81% inh., IC₅₀=6.8 μ M) with a pyridyl moiety (**78–80**) resulted in compounds with similar activity (73–86% inh.) and improved hydrophilicity. The 3-pyridyl substituted chroman-4-one (**79**) was the most potent inhibitor of the series with an IC₅₀ value of 3.7 μ M (86% inh.). In accordance with the modeling results, the pyridyl moiety of **79** has an optimal geometry to form a hydrogen bond with Gln142 which is located at the end of the hydrophobic channel. (Figure 16, b). The morpholine- and piperidine-substituted derivatives **86** and **87** exhibit only 17% and 40% inhibition, respectively. The heterocyclic moieties are basic and charged at pH 7.4 which seems unfavorable in the hydrophobic binding pocket.

Molecular modeling indicated that bicyclic substituents in the 2-position containing hydrogen bonding groups could potentially increase the potency. The chroman-4-one derivatives with the quinolinone and quinoline moieties (94a and 94b) were however, only moderate inhibitors with 59% and 56% inhibition, respectively. These results are consistent with the indolyl-substituted derivatives (37 and 38, Table 1) which had 53% inhibitory activity. The decreased activity indicates that there is limited space in the tunnel.

The tetrasubstituted chromones (Table 3) with an additional substituent in the 3-position (96–99), showed inhibitory activities between 50–81%. The acetamide 97 was the most potent inhibitor with 81% inhibition and an IC₅₀ value of 29 μ M. The introduction of a small heterofunctional side chain on the flat ring system did not result in the desired increase in potency via additional hydrogen bonding interactions.

Regarding the halogen bond interaction, we found that substitution of the chloride in the 6-position (**24** and **29**) with the stronger halogen bonding bromide (**27**, **100**, **109**) generally enhanced the potency (Figure 17).¹⁴⁸ This strengthens the hypothesis of a halogen bonding interaction between the halide in the 6-position and the backbone carbonyl of His187 (Figure 16).



Figure 18. The replacement of the Cl-group in the 6-position with stronger halogenbonding bromide increased inhibitory activity. "Synthesis described in Paper II, page 4.

A short summary of the SAR observed for the compounds synthesized in this chapter is given in Figure 19.



Figure 19. Illustrative summary of the SAR for the chroman-4-ones and chromone containing heterofunctional substituents.

3.3.5 Evaluation of antiproliferative properties of chroman-4-one based SIRT2 inhibitors

As described in section 1.2.4.2 is the enzymatic activity of SIRT2 associated with cancer and SIRT2 inhibitors have shown to possess antiproliferative effects in MCF-7 breast⁷⁴ and A549 lung cancer cells.⁷⁵ We therefore wanted to investigate whether our compounds could achieve similar effects. The pyridyl- and oxadiazole substituted chroman-4-ones **76** and **79**, two potent SIRT2 inhibitors with good physicochemical profiles were chosen for testing. The potent methyl ester-substituted derivatives were consciously omitted from testing due the risk for hydrolysis to the inactive carboxylic acid analogs. The human cancer cell lines A549 and MCF-7 were treated with increasing concentrations of **76** and **79** and the cell proliferation was measured using a sulforhodamine B assay determining the total protein mass which correlates to the number of cells.¹⁴⁹



Figure 20. SIRT2 inhibitors reduce A549 and MCF-7 cancer cell proliferation. The cells were treated with 0 to 100 μ M of 76 and 79. Cell proliferation was determined by a sulforhodamine B assay. The results are shown as mean ± SEM of two to three independent experiments. The asterisks indicate significant differences (* P<0.05, *** P<0.001 when compared to controls).

Both compounds showed a significant and dose-dependent inhibition of the cell proliferation in the two different cancer cell lines (Figure 20) which also correlated with their SIRT2 inhibitor activity. Carboxylic acid **70** was not able to achieve similar effects on the cell proliferation in the tested cancer cell lines.

We also wanted to examine the capability of **76** and **79** to achieve SIRT2 inhibition in a cell assay. Therefore, MCF-7 cells were treated with different concentrations of inhibitors and the acetylation state of lysine-40 of α -tubulin was assessed by western blot. As shown in Figure 21, **76** and **79** were able to inhibit the deacetylation of acetylated α -tubulin in a dose-dependent manner. These results reveal that the compounds can enter cells and decrease the deacetylation of a SIRT2 target protein.



Figure 21. The effects of SIRT2 inhibitors on α -tubulin acetylation. MCF-7 cells were treated for 6 h (left panel) or 18 h (right panel) with 40 nM trichostatin A plus indicated concentrations of 76 or 79. The results are shown as mean ± SEM of three independent experiments. The asterisks indicate significant differences (* P<0.05, *** P<0.001 when compared to controls). The representative Western blots are shown below.

Flow cytometric cell cycle analysis was performed in order to examine the basis of the antiproliferative effects of **76** and **79**. This analysis showed that treatment of the cells with 100 μ M **76** resulted in cell cycle arrest in the G₁/G₀ phase and a significant decrease in the fraction of cells in the S-phase (Figure 22). Treatment with 50 μ M **79** resulted in similar cell cycle arrest in A549 cells (significant) and MCF-7 cells (trend) (Figure 22). No apoptosis was observed in any of the samples (data not shown). The G₁/G₀ arrest is not necessarily resulting from increased acetylation of α -tubulin. It is likely that the studied compounds also have other effects than altering the α -tubulin acetylation status. However, G₁/G₀ arrest upon treatment with SIRT2 inhibitors has also been reported by others.^{150,151}



Figure 22. Effects of SIRT2 inhibitors on A549 and MCF-7 cell cycles. The cells were subjected to control treatment (0.5% DMSO) or treatment with **76** (50 μ M) or **79** (100 μ M) for 18 h. Flow cytometric analysis of DNA content was done after propidium iodide staining. Percentage of cells in each phase of the cell cycle (G₁/G₀, S, and G₂/M) is indicated. The results are shown as mean ± SEM of two to four independent experiments. The asterisks indicate significant differences (* P<0.05, ** P<0.01, *** P<0.001 when compared to controls).

3.3.6 Studies of the effect of SIRT2 inhibition on brain tumor cells

In collaboration with Helena Carén and co-workers from the Sahlgrenska Academy (University of Gothenburg), the effects of our SIRT2 inhibitors on brain tumor cells were investigated. Of these compounds, chromone **99** showed the most promising results in preliminary test. Treatment of pediatric and adult brain tumor stem cells with **99** induced a decrease in cell viability (Figure 23). The number of cells was reduced by 37–63% after 48 h of treatment. A higher reduction in brain tumor stem cells compared to neural stem cell (NSC) lines was observed.



Figure 23. The effect of 99 on the viability of brain tumor lines. Cells were cultured in NS media according to previous work and seeded in 96 wells plates. The pediatric (left panel) and adult (right panel) brain tumors were treated with 5 μ M of 99 for 48 h. The cells were stained with DAPI after fixation with PFA 4%. The plates were scanned by a High Throughput Screening System (Operetta, Perkin Elmer). Data analysis was done by Harmony software, Image J and Sigmaplot 11.0.

Furthermore, the morphology of the cells was investigated. The morphological image of brain tumor cells after treatment with **99** (Figure 24) showed a change in cell shape compared to untreated cells. Chromone **99** could reduce the number of elongated cells at a concentration of $5 \,\mu$ M.



Figure 24. Cell morphology of NCS and brain tumor cells before (left) and after the treatment with 99 (right). Cells were cultured in NS media according to previous work and seed in 96 wells plates and treated with 5 μ M solution of 99. FITC-Phalloidin staining was performed according to manufacturer's protocol (SIGMA). Data analysis was done by Harmony software, Image J and Sigmaplot 11.0.

Moreover, SIRT2 gene expression was detected in all samples analyzed as illustrated in Figure 25.



Figure 25. SIRT2 expression levels by qPCR in NSC and brain tumor lines. To assess the expression of SIRT2 gene, total RNA was obtained using Zymo kit, cDNA was generated by Superscript kit and amplified by using primers for human gene by Quantitative real-time PCR was performed in a BioRad CFX.

Further studies are needed, to clarify if the observed effect on the viability of the glioma cells is related to the SIRT2 inhibitory activity of **99**. In addition, the change in morphology needs to be investigated in more detail. Interestingly, the inactive carboxylic acid **70** had no effect on the viability or morphology of the control and glioma cells.

3.4 SUMMARY OF PAPER I AND II

In summary, we have shown that chroman-4-ones and chromones can serve as scaffolds for SIRT inhibitors with high potency and selectivity. The tested chroman-4-ones were synthesized using a base-promoted aldol condensation in varying yields. The method has been shown to be compatible with a variety of aldehydes carrying functional groups. Modification of chroman-4-ones carrying a terminal hydroxyl group enabled the introduction of e.g. carboxylic acid, amide or aliphatic heterocyclic groups. A small series of chromones derivatives substituted with an amine, nitrile or acetamide group in the 3-position were also synthesized. The initial SAR study in Paper I revealed a close relationship between the substituent in the 2-, 6-, and 8-position and the carbonyl group; Cl, Br and Me-groups on the aromatic ring as well as a pentyl or phenethyl group in the 2-position being most advantageous. However, the high lipophilicity of the compounds precluded evaluation in cellbased assays. Introduction of heterofunctional substituents in the 2-position (Paper II) furnished compounds with improved physicochemical properties with retained activity. Two compounds showed antiproliferative effects in a lung and a breast cancer cell line and a chromone-based inhibitor affected the viability and morphology in brain tumor cells. A homology model of SIRT2 based on a SIRT1 x-ray structure was built and docking studies proposed a binding mode of the chroman-4-one based inhibitors. The modeling studies contributed to gain a deeper understanding of the SAR data.

4 IDENTIFICATION OF THE BINDING SITE OF THE CHROMAN-4-ONE BASED SIRT2 INHIBITORS (PAPER III)

4.1 PHOTOAFFINITY LABELING

The principle of photoaffinity labeling (PAL) involves a photoactivatable but chemically stable ligand (probe) that upon radiation with light of a certain wavelength can form a highly reactive intermediate which irreversibly binds the ligand to the target. Photoaffinity labeling was first described in the 1960's by Westheimer and coworkers¹⁵² since then efforts have been put into the development of new photoreactive groups as well as into the analysis of the covalently joined complexes comprising biomacromolecules and the probes.

The technique can be a helpful tool to study biomolecular interactions like of proteinprotein/ligand interactions, identification of biomolecular targets and binding sites of bioactive compounds.^{153,154}

4.1.1 Photoreactive groups

The photoreactive group has to meet certain criteria to be useful for labeling studies of biological systems:¹⁵⁴

- It has to be chemically stable under the conditions where the biological system is stable.
- It should form reactive species only upon radiation.
- The generated reactive species should have a shorter half-life than the complex of the studied ligand and the biological target to avoid non-specific labeling.
- Photoactivation at wavelengths longer than 300 nm is beneficial in order to avoid damage of the biological system.
- The photoreactive group should not influence the biological activity of the ligand negatively.

The generation of the reactive species is usually accompanied with the release of a stable counterpart which is preferably a gaseous compound. So, the majority of the photoreactive groups release nitrogen gas as a result of irradiation. The azide, diazo, and diazirine groups (Figure 26) fall into this category. On the other hand, derivatives forming radicals upon irradiation can also be used as PAL probes; benzophenones are representative examples of this group (Figure 26).



Figure 26. Commonly used photoactivatable groups. Arylazides (left), trifluoromethylaryldiazirines (middle), and benzophenones (right).

4.1.1.1 Aryl azide

Aryl azides form singlet nitrenes upon radiation with wavelengths below 300 nm with a concomitant loss of N₂ gas. The singlet nitrene (101, Scheme 14) can either react with C-H and N-H bonds via insertion reactions, undergo a rearrangement by ring expansion to didehydroazepines (102) or form triplet nitrenes (103) via intersystem crossing (ISC). The didehydroazepine can be captured by nearby nucleophiles, or in the absence of nucleophiles polymerization will occur. Triplet nitrene will react as a diradical under hydrogen atom abstraction leading to radical like reactions.¹⁵⁵ The dominant reaction occurring upon photoactivation in solution at room temperature is the ring expansion leading to 102 which then is trapped by nucleophiles.¹⁵⁶ Thus, a reaction with the ketenimines relies on the presence of nucleophilic amino acids at the interaction site of the probe and the molecular target. This leads to a risk of irrelevant labeling because the long-lived ketenimines may exit the binding site if no nucleophilic capturing partner is present. However, ortho-fluorinated aryl azides are promising photolabeling probes as these have been shown to undergo ring expansion more slowly at room temperature.¹⁵⁷ This leaves enough time for the photogenerated singlet nitrene to participate in insertion reactions with amino acids located at the binding site.

Scheme 14. Formed reactive species upon photoactivation of aryl azides formed and their reaction pathways.



The main advantages of the aryl azides are their relatively small size and the easy preparation. This makes them attractive even though the activation occurs at wavelengths below 300 nm and the crosslinking efficacy might be low due to many reaction pathways after activation when non-fluorinated probes are used.¹⁵⁵

4.1.1.2 Diazirines

Diazirines were first proposed as reagents for photolabeling by Smith and Knowles in 1973.¹⁵⁸ The diazirines can be photoactivated using light with wavelengths typically between 350 and 365 nm. Upon irradiation the light sensitive group either undergoes photolysis to form a highly reactive carbene (**104**, Scheme 15) with loss of N₂, or it can photoisomerize to the diazo isomer (**105**).^{155,159} The diazo isomer can be converted to the carbene intermediate **104** under prolonged radiation at 350–365 nm. The highly reactive singlet carbene intermediate is responsible for efficient insertion into C-H, N-H, and O-H bonds. The singlet carbene can also form triplet carbene (**106**) which instead acts as a diradical.¹⁵⁵

Scheme 15. Formed reactive species upon photoactivation of diazirines formed and their reaction pathways.



The main advantages of diazirines are their small size, their activation at longer wavelengths (350-365 nm) minimizing the risk of damage of the biological target, their chemical stability, the short lifetime upon radiation and the high reactivity.¹⁶⁰ The high reactivity of the carbene species leads to quenching by surrounding water which minimizes non-specific labeling because only ligands in close vicinity to the binding site will covalently bind to the target molecule. However, the quenching leads to low crosslinking yields. Another drawback of the diazirines is the formation of the diazo intermediate. This long lived species could cause undesired labeling even in the dark due to side reactions via nucleophilic substitution.¹⁵⁸ However, introduction of a trifluoromethyl group reduces the amount of diazo isomer and increases the stability of the diazo compound towards nucleophilic side reactions.¹⁵⁹ This potentially minimizes the occurrence of non-specific labeling.

4.1.2 Identification of the photolabeled amino acid residues

A schematic overview of the photoaffinity labeling workflow is illustrated in Scheme 16. A ligand is incubated with the target protein and subsequent photoactivation by light of appropriate wavelength results in covalent binding of the probe to its biological target. Proteolytic digestion of the labeled protein forms small peptides which can by analyzed liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify the labeled amino acid (Scheme 16, **A**).¹⁵⁴ A reporter-free method needs highly efficient cross-linking to enable determination of the modified peptidic fragments.

Scheme 16. Schematic overview of a PAL approaches. (**A**) Tag-free approach. (**B**) Reporter-tag functionalized photo probe applied in PAL experiment. (**C**) Bioorthogonal strategy with post-introduction of the tag.



If the labeling efficiency is low, introduction of a reporter group can facilitate the detection of the labeled protein and also allow enrichment of the cross-linked fractionations of the biomolecule (**B**). Reporter tags that are commonly used include radioactive isotopes (¹²⁵I, ³H), fluorophores or biotin, the latter combines visualization and purification of the photo-cross-linked product.^{160,161} Under circumstances when the tag negatively alters the biological activity of the probe, a bioorthogonal approach (**C**) can be used. This method comprises of a post-introduction of the reporter group into the PAL probe via a ligation handle.¹⁶² The most frequently used methods for incorporation of the tags after cross-linking are the Staudinger-Bertozzi ligation¹⁶³ and the copper-catalyzed azide-alkyne cycloaddition.¹⁶⁴ An alkyl azide or terminal alkyne moiety in the PAL probe usually acts as handle for the attachment of the reporter tags.¹⁶²

To identify the labeled amino acid as mentioned above, the sample is subjected to mass spectrometric analysis (MS).^{154,165} Edman degradation for peptide sequencing is rarely used

nowadays. Peptide sequencing performed by LC-MS/MS has become the tool of choice for identification of ligand-protein interaction sites.

PAL has been successfully applied to study biological targets,¹⁵³ e.g. a diazirine-based PAL probes have been used to identify the receptor-binding site of propofol, a commonly used anesthetic,¹⁶⁶ and to map active site amino acids of a tyrosine phosphatase B.¹⁶⁷ By applying a cross-linkable version of a HDAC8 inhibitor, an unknown binding pose of this compound could be discovered.¹⁶⁸ Recently, Kalesh *et al.* reported a SIRT5-specific benzophenone containing PAL probe based on a succinylated peptide to enable studies of SIRT5-function and the post-translational succinylation.¹⁶⁹

4.2 IDENTIFICATION OF THE BINDING SITE OF THE CHROMAN-4-ONE BASED SIRT2 INHIBITORS USING PHOTOAFFINITY LABELING

In order to investigate the binding site of the chroman-4-one-based SIRT2 inhibitors a photoaffinity labeling approach was chosen. As only one crystal structure of SIRT2 was solved⁴³ at the start of this project we anticipated that crystallizing SIRT2 with our inhibitor might be too challenging.

4.2.1 Design and Synthesis of PAL Probes for SIRT2 based on chroman-4-one Scaffold

The incorporation of the photoreactive group into the rigid scaffold rather than incorporation in the flexible side chain in the 2-position was believed to most precisely mark the inhibitor-enzyme interaction site. The SAR study carried out on the chroman-4-ones (section 3.2.3) disclosed a small tolerance for large substituents on the aromatic part of the scaffold and that the 6-position contributes more to the activity than the group in the 8-position. Therefore, the photoreactive group was intended to be introduced in the 6-position and an azide and diazirine moieties were chosen prior to the bulkier benzophenone functionality (Figure 27).



Figure 27. Photoactivatable probes based on SIRT2 inhibitor 24.

Compound **107** was synthesized as outlined in Scheme 17 in analogy to previously reported procedures.^{153,170} 5'-Bromo-2'-hydroxyacetophenone was reacted with hexanal to yield 6-bromo-2-pentyl-chroman-4-one (**109**). Reduction of the carbonyl group in **109** with NaBH₄ yielded **110**. Subsequent protection of the hydroxyl group as a silyl ether using TBDMSCl and imidazole afforded **111**. Chroman-4-ol **111** was reacted with *n*-BuLi at -78 °C followed by the addition of ethyl trifluoroacetate to introduce the trifluoroacetyl group (**112**).

Heating of **112** with hydroxylamine hydrochloride in pyridine at 70 °C produced oxime **113** in 90% yield. The oxime was directly tosylated with tosyl chloride in the presence of triethylamine and DMAP in CH_2Cl_2 to produce **114**. For the formation of the diaziridine, a solution of **114** in CH_2Cl_2 was added to liquid ammonia at -78 °C.



aReagents and conditions: (a) Hexanal, DIPA, EtOH, MW, 170 °C, 1 h; (b) NaBH₄, MeOH/THF, rt, 15 min.; (c) TBDMSCl, imidazole, CH₂Cl₂, rt, 18 h; (d) *n*-BuLi, ethyl trifluoroacetate, THF, -78 °C \rightarrow rt, 18 h; (e) NH₂OH·HCl, pyridine, 70 °C, 16 h; (f) TsCl, Et₃N, DMAP, CH₂Cl₂, 0 °C \rightarrow rt, 5 h; (g) NH₃₍₀₎, CH₂Cl₂, -78 °C \rightarrow rt, overnight; (h) I₂, Et₃N, CH₂Cl₂, rt, 2 h; (i) TBAF, THF, rt, 3 h; (j) (COCl)₂, DMSO, Et₃N, THF, -78 °C \rightarrow rt.^{*b*}Isolated yield over three steps.

The formed diaziridine **115** was oxidized to the diazirine (**116**) using iodine and triethylamine in CH_2Cl_2 . Finally, deprotection of **116** with TBAF in THF at room temperature and a subsequent Swern oxidation of the hydrolxyl group provided the photoaffinity probe **107** in 80% yield.

The synthesis of the arylazide probe **108** is shown in Scheme 18. The reaction sequence starts with the reduction of the nitro group of **30** by catalytic hydrogenation in the presence of 10% Pd/C at room temperature to furnish **118**. The amine was converted to the desired azide **108** using *t*-BuONO and TMSN₃ as reported by Barral *et al.*¹⁷¹

Scheme 18. Synthesis of the azide-substituted 2-pentylchroman4-one 108.^a



^{*a*}Reagents and conditions: (a) H₂, 10% Pd/C, EtOH, rt, overnight; (b) *t*-BuONO, TMSN₃, MeCN, 0 °C \rightarrow rt, 1 h.

4.2.2 Investigation of the SIRT2 inhibition of the PAL probes

Photoprobes **107** and **108** were evaluated for their inhibitory effect on the activity of SIRT2 using the fluorescence based assay (Figure 28). Arylazide **108** showed only a modest 51% inhibition at 200 μ M concentration (Figure 28, **A**). However, diazirine-substituted chroman-4-none **107** inhibited the SIRT2 activity with 78% at the same concentration. The IC₅₀ value of **107** was determined to 8.2 μ M (Figure 28, **B**). Probe **107** displayed a similar potency as our previously reported inhibitors (Table 1 and Table 2) and it was therefore chosen for the PAL studies.



^{*a*}SD, standard deviation (n=2).

Α

Figure 28. (A) Inhibition of SIRT2 at 200 μ M concentration of chroman-4-ones 107 and 108. (B) IC₅₀ determination of diazirine 107 (IC₅₀=8.2 μ M).

4.2.3 Investigation of the photochemical properties of diazirine 107

Before the PAL experiments were performed, the photochemical properties of **107** were investigated using UV/VIS and ¹H and ¹⁹F NMR spectroscopy. A methanolic solution of **107** was irradiated with a high-power UV-LED ($\lambda = 365$ nm, light flux: 23 mW/cm²) during which UV-VIS absorption spectra were recorded at fixed time points. The results are shown in Figure 29. Consistent with spectral data reported for other aryl diazirines,^{159,172} **107** showed the characteristic diazirine absorption around 360 nm (Figure 29). Upon UV-irradiation of the sample, a clear decay of the diazirine absorbance band was observed which is attributed to the photodecomposition of **107**. The disappearance of the absorption at 360 nm proceeded concurrently with an absorption change at 275 nm, where a rapid increase (for t = 0-20 s) was followed by a decrease. This change in absorption can be ascribed to the partial rearrangement of the diazirine to the diazo isomer (**119**) which upon further irradiation undergoes photolysis resulting in a subsequent decay of the absorption at 275 nm.^{159,172} No further change in absorption was observed after a radiation time of 9 min (dashed line).



Figure 29. Absorption spectra of **107** in MeOH following irradiation with 365 nm light. A 0.2 mM solution of **107** in MeOH (bold line) was irradiated for t = 5, 10, 20, 30, 45, 60, 90, 120, 150, 180, 240, 360, 540 s (dashed line). The spectra clearly show the photolysis of the diazirine moiety at 360 nm.

The course of the photolysis was further examined using reversed-phase HPLC. Aliquots were taken at different time points and subjected to HPLC analysis (Figure 30). The results are in agreement with the observations from the UV-VIS experiment.



Figure 30. HPLC analysis of diazirine **107** in MeOH upon irradiation with 365 nm light. Samples taken after t = 0, 10, 30, 60, 120, 150, 360, 540 s UV-irradiation. Integration of the absorption peaks from the eluting compounds (UV-detection at 254 nm) shows the rapid photo-conversion of **107** (squares) into the insertion product **120** (triangles) *via* formation of **119** (circles). The consumption of **107** (solid line) and production of **120** (dotted line) can be fitted to first-order exponentials ($\tau = 5.1$ s and 19 s respectively), while a biexponential fit gives $\tau = 12$ s and 50 s for the rise and decay of **119** (dashed line).

In addition to the UV-VIS and HPLC analyses, the photolysis of **107** was also investigated by ¹H and ¹⁹F NMR spectroscopy in CD₃OD which is shown in Figure 31. The light-dependent decomposition and insertion of **107** into the O–D bond of the solvent are clearly shown by the disappearance and build-up of proton signals in the aromatic region (Figure 31, **A**). The ¹⁹F NMR spectra show the appearance of three new signals together with the fast decay of the starting material (Figure 31, **B**). The light-dependent decay of the higher shift signal was ascribed to diazo isomer. As a result of the formation of an additional stereocenter through insertion into CD₃OD two signals corresponding to the product appeared in the ¹⁹F spectrum. The light-induced reaction of **107** with deuterated cyclohexane showed similar results as those with CD₃OD.



Figure 31. Photolysis of a 2 mM solution of **107** in CD₃OD was followed by ¹H and ¹⁹F NMR spectroscopy at time points t=0, 10, 20, 30 and 60s. (A) Expanded view on the aromatic proton signals and (B) the fluorine signals.

4.2.4 Photoaffinity labeling of SIRT2

The binding of the probe to SIRT2 was intended to be studied with the tag-free method illustrated in **A** of Scheme 16 with LC-MS/MS analysis after tryptic digestion to determine the site of modification. As mentioned in section 1.2.3 does the enzyme undergo conformational changes upon NAD⁺ and peptide substrate binding. However, we have no knowledge if the chroman-4-one binds to the apo-enzyme or if the binding pocket is generated upon co-substrate binding. Therefore, recombinant SIRT2 was incubated with varying concentrations of **107** in the presence or absence of NAD⁺ and/or the peptide substrate. The experimental set-up for the experiment is shown in Figure 32. After incubation for 5 min at 37 °C, the samples were irradiated with a high-power UV-LED ($\lambda = 365$ nm) for 3×10 s with a 30 s resting periods. The samples were subjected to tryptic digestion followed by LC-MS/MS analysis. SIRT2 was observed with a sequence coverage of 90% (Paper III, Supporting Information, Figure S2).



Figure 32. Experimental set-up of the PAL experiment. The sample of SIRT2 and photoprobe 107 was placed in a Eppendorf vial placed 2 cm from the UV-Led and covered with a box during the irradiation.

In an experiment at an inhibitor concentration of 400 µM without NAD⁺ and peptidesubstrate the tryptic peptide with the amino acid sequence IAGLEQEDLVEAHGTFYTSHCVSASCRHEYPLSWMK (m/z 733.00989⁶⁺, Figure 33) was detected with an mass of 298.11807 Da greater than the parental peak. This difference corresponds to the molecular weight of 107 with loss of N₂. The data acquired for this mass ion could not be matched other proteins in a SwissProt database search. Data analysis of the MS2 fragmentation of this peptide showed that the modification was located at one of the amino acids between Q180 to H187 (underscored in peptide sequence in Figure 33, A). Fragment ions of the peptide precursor, with inhibitor cross-linking at H187, are shown in the MS/MS spectra in Figure 33 (B). For the peptide sequences Q175 to E179 as well as for G181 to K210 multiple +1 to +4 charged b- and y-ions were observed and clearly exclude inhibitor binding to these amino acids. However, due to the quality of the MS2 data obtained from the ¹⁸⁰QEDLVEAH¹⁸⁷ section, no distinct cross-linked amino acid could be identified.

An alternative explanation for the identification of the ¹⁸⁰QEDLVEAH¹⁸⁷ sequence as the potential cross-linking site could be that **107** is attached to several of these amino acids. Single modifications of the amino acids from Q180 to H187 will result in the same precursor ion mass. The b and y-ions from each of these modified peptides will however be very weak and might be difficult to detect. Analysis using chymotrypsin for protein digestion to obtain smaller tryptic peptides of this region and trials with MS3 detection was performed without any additional improvement of the results.

The observed cross linking yield in the experiment was extremely low which complicated the mass analysis. Low-abundant cross-linked peptides co-eluted with other dominant unmodified peptides and therefore became discriminated in the data-dependent LC-MS analysis (Paper III, Supporting Information, Figure S4). This also most likely explains the observed lack of modified tryptic peptides and product ions in MS2 in some experiments.



B



Figure 33. (**A**) Amino acids observed as single and double charged mass ions. Sequence of A to Y is covered by multiple charged b and y fragments. (**B**) NanoLC-MS/MS spectra of the protonated precursor m/z 733.00989, and charge equal to six, eluting at 47.9 min. Peptide sequence IAGLEQEDLVEAHGTFYTSHCVSASCRHEYPLSWMK with a 298.11807 Da modification at H187 was identified using a mass error tolerance of 5 ppm.

Using our SIRT2 homology model and the x-ray structure of the apo-enzyme, the ¹⁸⁰QEDLVEAH¹⁸⁷ region was visualized (Figure 34). Docking studies showed that **4** can bind to the C-pocket of SIRT2 in a similar way as observed for other chroman-4-one-based SIRT2 inhibitors (Figure 34). The diazirine moiety is directed towards H187 and could form an insertion product with this amino acid. The amino acids on the *N*-terminal site of H187 are located in a helix pointing towards the surface. Photo-cross linking of the probe to Q180 to D182 is likely as these amino acids are located on the surface of the enzyme. However, the ¹⁸³LVEA¹⁸⁸ stretch is not accessible for the probe as these amino acids are buried in the interior of the enzyme.



Figure 34. (A) Positioning of the eight amino acids (QEDLVEAH) that are labelled by the photoprobe in the SIRT2 homology model. The van der Waals or solvent accessible surfaces of the amino acids are shown. The red highlighted regions are exposed to the solvent and the amino acids Gln180, Glu181 and His187 positioned in these areas would therefore be most accessible for binding of the photoprobe. (B) Inhibitor 107 bound in the C-pocket of SIRT2. The diazirine function is pointing towards His187. The van der Waals or solvent accessible surface of the C-pocket is shown.

As mentioned earlier both NAD⁺ and the peptide substrate can induce conformational changes of the enzyme upon binding. The experiment in which the modified peptide sequence was identified was however performed without NAD⁺ and peptide substrate present. Therefore, the SIRT2 structure in the PAL experiment might differ from the one observed in the homology model. However, recently Moniot *et al.* reported an x-ray structure of SIRT2 in complex with ADP-ribose.⁵⁰ The bound ligand mimicked NAD⁺ binding and SIRT2 adopted a similar conformation as observed in SIRT/NAD⁺ crystals. Interestingly, it was observed that the C-pocket, which we proposed as potential binding site for our chroman-4-one based SRTI2 inhibitors, appeared to be pre-formed in the apo-structure of the enzyme⁵⁰ Therefore, it can be assumed that the probe is able to interact with SIRT2 in the C-pocket also in the apo-enzyme.

4.2.5 Summary of Paper III

Two potential PAL probes based on the chroman-4-one scaffold with a diazirine and an azide moiety have been synthesized. Evaluation of their SIRT2 inhibitory activity revealed **107** to be the more potent inhibitor with an IC₅₀ value of 8.2 µM. The photochemical properties of **107** were investigated using UV-VIS and NMR spectroscopy. PAL experiments with SIRT2 followed by tryptic digestion and LC-MS/MS analysis located the site of attachment to the amino acid sequence ¹⁸⁰QEDLVEAH¹⁸⁷ present in the tryptic peptide IAGLEQEDLVEAHGTFYTSHCVSASCRHEYPLSWMK. This stretch is located close to the active site of SIRT2. Unfortunately, a low cross-linking yield complicated the identification of the specific amino acid(s) modified by the probe.

5 A SCAFFOLD REPLACEMENT APPROACH TOWARDS NEW SIRT INHIBITORS (PAPER IV)

5.1 SCAFFOLD

For a long time our research group has focused on the use of chromone and chroman-4-ones as scaffolds for the development of biologically active compounds. The term scaffold is used to describe the core structure of a molecule.¹⁷³ This central element is substituted with groups representing the key features to achieve a desired biological activity (Figure 35).¹⁷³ The core structure is mainly represented by rigid and cyclic frameworks which are decorated with substituents allowing an optimal interaction with biological targets.



Figure 35. Illustration of ligand binding with a target. Extruding side chains from the core structure allow an optimal interaction with the target.

The replacement of the scaffold in a bioactive compound with another core structure can be desirable for several reasons e.g. decrease in lipophilicity to increase solubility, increase of metabolic stability or decrease of flexibility to facilitate improved binding.¹⁷⁴ The new scaffold should show space similarities with the parent scaffold and be able to arrange the substituents in such way that key interactions with the biological target remain to ensure retained activity.¹⁷⁴

5.1.1 Quinolones and cyclic sulfonamides

Quinolin-4(1*H*)-ones and bicyclic secondary sulfonamides like saccharins or benzothiadiazine-1,1-dioxides are versatile scaffolds found in bioactive compounds. Quinolin-4(1*H*)-one (Scheme 20) is structurally similar to the chromone and is a common scaffold in broad spectrum antibacterial agents such as fluoroquinolones.¹⁷⁵ Bicyclic sulfonamides like saccharins or benzothiadiazine-1,1-dioxides (Figure 36) are versatile scaffolds found in bioactive compounds. Benzothiadiazine-1,1-dioxides have been used as diuretic drugs since the 1950s, e.g. in the treatment of hypertension.¹⁷⁶ In recent years, nootropic and neuroprotective effects of substituted benzothiadiazine-1,1-dioxides have implicated a potential use as drugs in memory and learning disorders.^{177,178} Derivatives with this scaffold also possess antiviral properties.¹⁷⁹ Saccharin (Figure 36), widely used as an artificial sweetener, has been successfully used as a core structure for e.g. inhibitors of

carbonic anhydrase IX¹⁸⁰ and is a key element of repinotan, a highly selective 5-HT1A-receptor agonist.¹⁸¹



Figure 36. Chemical structure of benzothiadiazine 1,1-dioxides and saccharin.

The most commonly used synthetic procedures for the synthesis of saturated benzothiadiazines involves an acid-catalyzed reaction of a 2-aminobenzenesulfonamide and an aldehyde via an imine intermediate which upon an intramolecular cyclization forms the saturated scaffold (Scheme 19, **A**).^{177,182} Unsaturated benzothiadiazines are commonly obtained by amide coupling of 2-aminobenzenesulfonamide with an activated carboxylic acid followed by a subsequent cyclization/dehydration step (**B**).¹⁸³ The saccharin scaffold (Scheme 19, **C**) can be prepared via oxidation of *ortho*-methyl-benzsulfonamides (route 1).^{184,185} Alternatively, the scaffold can be formed via a Diels-Alder-reaction (route 2),¹⁸⁶ or via *ortho*-lithiation of benzsulfonamides followed by ring closing (Scheme 19route 3).^{187,188}

Scheme 19. Retrosynthetic outline of the synthetic methods towards 3,4-dihydro-2H-[1,2,4]benzothiadiazine-1,1-dioxide (A), 3H-[1,2,4]benzothiadiazine-1,1-dioxide (B) and saccharin (C) frameworks.



The majority of the most potent SIRT2 inhibitors based on the chroman-4-one scaffold are rather lipophilic and showed unsatisfactory water solubility. The SAR studies revealed that non-polar substituents are mandatory to achieve potent compounds. Although we were able to improve hydrophilicity by incorporation of more polar substituents, we were interested to study whether more hydrophilic scaffolds could serve for the development of SIRT2 inhibitors. Therefore, we wanted to replace the chroman-4-one scaffold with other heterofunctional core structures shown in Scheme 20.





From the SAR around the chroman-4-ones it was known that halogens on the aromatic ring had beneficial effects on the SIRT2 inhibitory activity. If the novel scaffolds have a similar binding mode a bromide in the corresponding position would be desirable. We also envisioned to introduce substituents corresponding to those used in the 2-position of potent chroman-4-ones.

5.2 SYNTHESIS OF SCAFFOLD ANALOGS OF THE CHROMAN-4-ONES

5.2.1 Synthesis of trisubstituted quinolin-4-(1H)-one analogs

The quinolin-4-(1*H*)-ones (**123a–c**) were synthesized from β -ketoesters (**121a–c**) and an aniline employing the Conrad-Limpach reaction (Scheme 21). Compounds **121a–c** were obtained in good yields by reacting monomethyl potassium malonate and carboxylic acids with CDI in anhydrous THF at room temperature according to a reported method.¹⁸⁹ 2-Bromo-4-chloroaniline was then reacted with **121a–c** under argon at 50 °C for 48 h to afford the intermediate enaminoesters **122a–c**.



^{*a*}Reagents and conditions: (a) CDI, MgCl₂, THF, rt, 16 h; (b) 2-Bromo-4-chloroaniline, *p*-TSA, neat, 50 °C, 48 h; (c) i. Cyclohexane, reflux, 30 min; ii. Ph₂O, 250 °C, 45 min, MW; (d) K₂CO₃, MeI, DMF, rt, 4–6 h.^{*b*}Isolated yield over two steps.

Scheme 21. Synthesis of quinolin-4-(1H)-one derivative 123a-c and 124a-c.ª

After removal of excess aniline and *p*-TSA the cyclization was achieved by heating the enaminoesters in diphenyl ether to 250 °C for 45 min using microwave heating. The quinolin-4-(*1H*)-ones (**123a–c**) were finally isolated by crystallization from hexane.[§] To investigate the importance of the NH-group for activity, **123a–c** were methylated using MeI in DMF providing **124a–c** in good yields.¹⁹⁰

5.2.2 Synthesis of derivatives based on the benzothiadiazine-1,1-dioxide scaffold

The benzothiadiazine-1,1-dioxide scaffolds (Scheme 22) were formed by the reaction of **125** with aldehydes or carboxylic acids which defined both the substituent in the 3-position and the degree of saturation of the scaffold. The dibrominated **125** was obtained by reacting 2-amino-benzenesulfonamide with Br₂ in DMF at room temperature. Benzothiadiazine-1,1-dioxide **127a-d** were prepared in 34–53% yield via an acid catalyzed reaction of **125** with aldehydes **93b** and **126a-c** under microwave heating at 120 °C in dioxane. Aldehydes **126a-b** were commercially available whereas aldehydes **93a** and **126c** were synthesized. The synthesis of **93a** is described in Scheme 12. 3-(3-Bromophenyl)propanal **126c** was synthesized via a reduction of 3-(3-bromophenyl)propionic acid with BH₃·SMe₂ in THF at room temperature followed by a Swern oxidation in 73% overall yield.

Scheme 22. Synthesis of the saturated and unsaturated benzothiadiazine 1,1-dioxide 127a-d and 129a-c.^{*a*}



aReagents and conditions: (a) Br₂, DMF, 10 °C \rightarrow rt, 22 h; (b) Appropriate aldehyde, 4 M HCl in dioxane, 120 °C, MW, 1–2.5 h; (c) i. Appropriate carboxylic acid, CDI, CH₂Cl₂, rt, 1.5 h; ii. **125**, CH₂Cl₂, DMF, reflux, 21 h; (d) Cs₂CO₃, EtOH, 120 °C, 1.5 h, MW. *b*Isolated yield over two steps.

The synthesis of **129a–c** (Scheme 22) was accomplished via a reaction of CDI-activated carboxylic acids with **125** leading to amide intermediates **128a–c**. Cyclization of **128a–c** in a microwave heated reaction with Cs_2CO_3 in EtOH at 120 °C. Precipitation of the final products acidification yielded **129a–c** in 20–37% yield. As these derivatives however showed poor solubility in most solvents, the synthesis of other derivatives was not continued.

[§]Compounds 123a-c were provided by Dr. Kristian Meinander, University of Helsinki, Finland.

5.2.3 Synthesis of saccharine derivatives

As earlier mentioned the SAR study of the chroman-4-ones showed that halogen atoms in the 6- and 8-position of this scaffold are essential for activity. Therefore, it was believed that 4,6-dihalogenated saccharins would have the ability to show SIRT2 inhibitory activity.

The saccharins were planned to be synthesized via oxidative cyclization of 2toluenesulfonamides. The attempt to obtain a dihalogenated precursor for saccharin synthesis via chlorosulfonation of 4-bromo-2-chlorotoluene yielded only the non-desired regioisomer (**131**) which had earlier been observed by others.¹⁹¹ Also the attempt to dibrominate 2methylbenzenesulfonamide by the previously used method (Br₂ in DMF) was unsuccessful. As the substituent in the 6-position is most crucial for activity, the mono-brominated derivative **130** was instead synthesized by chlorosulfonation of 4-bromotoluene. The desired regioisomer was obtained as the major product together with small amounts of the other regioisomer (14–21% according to ¹H NMR analysis.) The isomeric mixture was taken further and **130** was converted to the corresponding sulfonamide **132a** with aqueous ammonia. For the synthesis of the saccharin scaffold the method reported by Xu *et al.* using the CrO₃ /H₆IO₅ oxidation system was used.¹⁸⁴

Scheme 23. Synthesis of 6-bromo-saccharin 135.^a



^{*a*}Reagents and conditions: (a) ClSO₃H, CH₂Cl₂, 0 °C \rightarrow rt, overnight; (b) NH₄OH, Et₂O, 0 °C \rightarrow reflux \rightarrow rt, 48 h; (c) *tert*-BuNH₂, Et₃N, Et₂O, 0 °C \rightarrow rt, 33 h, 83% (d) **135** from **132a**: HI₅O₆, CrO₃, MeCN, reflux, 20 h, 46%; **135** from **132b**: HI₅O₆, CrO₃, Ac₂O, MeCN, 0 °C \rightarrow rt, 20 h; (e) TFA, reflux \rightarrow rt, 4 d, 62% over two steps.

6-Bromosaccharin (135) was obtained in only 46% yield. Xu *et al.* reported higher yields for the synthesis of *N-tert*-butyl substituted saccharins (133), hence this approach was tested. Oxidation of the *N-tert*-butyl-substituted sulfonamide 132b gave under similar reaction conditions 135 in an overall yield of 62% after removal of the *N-tert*-butyl group via heating of 133 to reflux in TFA. Thus, the second pathway gave 135 in higher yield, however the final deprotection of 133 proceeds very slowly on a gram scale.

Commercially available saccharin 134 and 6-bromosaccharin 135 were transformed to their corresponding sodium salts using NaOMe in MeOH and were further subjected to microwave-heated substitution reactions with a variety of alkyl halides to yield the N-substituted derivatives 138a-d and 139a-e in good yields.

Scheme 24. Synthetic procedures for the synthesis of *N*-alkylated saccharins 138a-d and 139a-e.^{*a*}



^aReagents and conditions: (a) NaOMe, MeOH, 6 h, room tem., >99%; (b) Alkyl halide, DMF, MW, 145 °C, 15 min. or 1 h.

5.3 BIOLOGICAL EVALUATION AND STRUCTURE-ACTIVITY RELATIONSHIP STUDY OF THE NEW SCAFFOLD ANALOGS

5.3.1 Evaluation of the inhibitory activity towards SIRT1-3

The synthesized compounds were evaluated for their SIRT2 inhibitory potency with the standard fluorescent based assay. Several representatives from each scaffold series were tested also for their inhibitory activity towards the SIRT1 and SIRT3 isoform. The results are summarized in Table 5.

5.3.2 Structure-activity relationship study

In general, the quinolone, benzothiadiazine-1,1-dioxide and the saccharin based compounds were less potent and selective inhibitors of SIRT2 than inhibitors based on chromone or chroman-4-one scaffolds.

Replacement of the chroman-4-one/chromone core with the nitrogen-containing quinolone equivalent significantly reduced the activity. The pentyl-substituted quinolone analog **123a** showed only 26% inhibition at 200 μ M concentration, compared to chromone **43** with 82% inhibition. Two slightly modified analogs (**123b** and **c**) of the phenethyl- and methyl ester-substituted chroman-4-ones, **68** (90% inh.) and **36** (81% inh.) were moderately active with 58% and 53% inhibition, respectively. *N*-Methylation resulted in poorly active compounds (**124a**-**c**) ranging from 31% to 11% inhibition.

The 3,4-dihydrobenzothiadiazine derivatives 127a-d showed the most active series of SIRT2 inhibitors with 48–74% inhibition. The most potent compound with 74% inhibition was **127c** carrying a terminal quinolone moiety in the R² side chain; being also more active than its chroman-4-one analog (**94a**, 51% inh.). The pentyl- and phenethyl derivatives (**127a** and **b**) showed roughly 50% inhibition.

Table 5. SIRT2 inhibitory activities of all as well as SIRT1 and SIRT3 inhibitory activities of selected scaffold analogs 12a–d and 15a–c.

					CI R^2 R^2				
		123a-c					124a-c		
No	R ²	Inh	ibition (%)) <i>a,b,c</i>	No	\mathbb{R}^2	Inh	ibition (%) <i>a,b,c</i>
110.	, , , , , , , , , , , , , , , , , , ,	SIRT1	SIRT2	SIRT3	110.		SIRT1	SIRT2	SIRT3
123a	, , ,	n.d.	26±1.4		124a	`**~~~	n.d.	26±0.9	n.d.
123b		7.6±2.2	58±2.1	41±3.0	124b		1.8±3.2	31±1.2	7.2±2.1
123c		n.d.	53±1.1		124c		n.d.	11±1.6	n.d.
$Br \xrightarrow{O} S^{O} S^{O} H$					$Br \xrightarrow{O} S \xrightarrow{O} Br \xrightarrow{N} R^{3}$ Br 129a-c				
No	R3	In	hibition (⁰ /0) ^{a,b,c}	No	R3	Inl	hibition (%	$(0)^{a,b,c}$
	K	SIRT1	SIRT2	SIRT3	140.	í.	SIRT1	SIRT2	SIRT3
127a		4.4±2.8	59±4.6	5.8±3.4	129a		n.d.	15±3.1	n.d.
127b	·*	4.6±4.2	48±1.7	54±9.2	129b	Br	0±3.7	24±0.8	3.2±2.0
127c	Br	9.7±3.9	59±0.4	9.9±1.5	5 129c	Br	n.d.	10±1.5	n.d.
127d	-second second s	25±1.6	74±1.3	28±1.5					
					Br S N-R				
		Inh	ibition (%	a,b,c			Ini	nibition (%	a,b,c
No.	R ³	SIRT1	SIRT2	SIRT3	No.	R ³	SIRT1	SIRT2	SIRT3
138a	×	n.d.	34±2.5	n.d.	139a	, the second sec	3.8±4.3	4.7±3.5	1.6±2.2
138b	, All and All	14±6.3	34±2.8	14±2.8	139b	Br	n.d.	15±1.9	n.d.
138c	- Et	8.7±3.4	13±5.0	7.2±1.9	139c	` ^{\$} _OH	5.2±4.0	12±4.8	15±5.6
138d	- E - N	17±4.3	12±4.9	14±2.4	139d	NO2	n.d.	6.2±4.2	n.d.
					139e	N N N N N N N N N N N N N N N N N N N	n.d.	13±4.4	n.d.

^aSD, standard deviation (n=3). ^bInhibition at 200 µM inhibitor concentration. ^cn.d.=not determined

Introduction of a bromide in the *meta*-position (**127b**) had no effect on the activity, however it increased the selectivity for SIRT2 over SIRT3 The unsaturated analogs 129a-c had no significant inhibiting activity against SIRT2.

To use the substitution pattern for the chroman-4-ones in the saccharin series was not tolerated and provided derivatives showing less than 35% inhibition of SIRT2 (Table 5, lower section). The most active of the tested compounds were the *N*-pentyl- and phenethyl substituted saccharins **138a** and **138b** with 34% inhibition. Interestingly, in contrast to the chroman-4-one-based inhibitors, a bromide group on the saccharin had no positive effect on the inhibitory activity.

Interestingly, **123b** and **127b** showed increased SIRT3 inhibitory activity not observed for the chroman-4-ones.

Docking studies were conducted to get an understanding of the reasons behind the different activity and selectivity profiles of the new scaffolds. A docking study of **127b** into the SIRT2 homology model (Figure 37) reveals the same key ligand-enzyme interactions as observed for the chroman-4-ones. However, docking studies indicate an unfavourable interaction of one oxygen atom of the sulfonyl group with an aspartic acid residue in the enzyme. Repulsion of these two groups could give rise to the decreased inhibitor activity (Figure 37). However, there might also be a possibility that the scaffold analogs have a different binding mode compared to the chroman-4-ones.



Figure 37. Two orthogonal views of **127b** bound in the C-pocket of SIRT2. The vdW or solvent accessible molecular surface of the C-pocket shows hydrophobic (green), hydrogen bonding (purple) and mild polar areas (blue). Sulfonamide **127b** can adopt a similar binding mode as the chroman-4-ones with key interactions present such as the halogen bond between the bromide in the 7-position and the backbone carbonyl of His187 and the hydrogen bond with the conserved structural water molecule (W17). The pentyl chain is buried in the hydrophobic channel (left). However, one of the oxygen atoms of the sulfonyl is positioned in a hydrophobic environment close to Asp170 which presumably can repel the ligand (side view, right). The NH-group in **127b** is located in a hydrophobic region (Leu103 and 138) where polar substituents are highly unfavorable (left). Increasing the lipophilicity via *N*-methylation is not desirable as the pocket is rather narrow.
5.3.3 Summary of Paper IV

The studies of different scaffold analogs have shown that it is challenging to replace the chroman-4-one scaffold with other more hydrophilic bicyclic frameworks to furnish potent SIRT2 inhibitors. The 3,4-dihydrobenzothiadiazine is the most promising of the studied novel scaffolds. Only five compounds were studied in this series and all of them showed at least ca. 50% SIRT2 inhibition. When considering the fact that even small changes in the chroman-4-one scaffold drastically changed the activity, it seems likely that more thorough studies on this scaffold could result in compounds with improved activity. Another interesting observation of the new series of compounds is the increased SIRT3 inhibitory activity with **127b** being the most active compound. This could open up for development of SIRT3 inhibitors based on the 3,4-dihydrobenzothiadiazine scaffold. A thorough investigation using molecular modeling is needed to get a deeper understanding of the structural requirements for the SIRT3 inhibition.

The thesis describes the synthesis of substituted chroman-4-ones and chromones using different efficient synthetic strategies. Biological evaluation of their SIRT inhibitory activity revealed a series of potent and selective SIRT2 inhibitors with low micromolar IC₅₀ values. A binding site was proposed using a homology model of SIRT2 which is consistent with acquired SAR. Efforts were directed to further evaluate the putative binding site using photoaffinity labeling. This approach identified eight amino acids including a conserved histidine residue positioned in the NAD⁺ binding site. Scaffold analogs of the chroman-4-ones/chromones were also successfully synthesized. The biological evaluation showed a decreased SIRT2 inhibitory effect. In addition, an increase in inhibitory activity against SIRT3 was observed for some derivatives.

For the future, the following issues would be of interest to investigate:

- The order of binding and potential competition between the binding of the chroman-4-ones and peptide substrate and/or NAD⁺ needs to be determined. It could be done using e.g. isothermal titration calorimetry (ITC). The obtained results would be helpful to refine the homology model and are also important for the design of new PAL experiments. Moreover, the SIRT2-selectivity of the inhibitors over SIRT1/3 should be investigated in detail to enable to development of also SIRT1 and SIRT3selective inhibitors. The compounds should also be tested for their effect on other NAD⁺ dependent enzymes, e.g. PARP1. Lastly, follow-up studies are needed to elucidate the underlying mechanism for the antiproliferative effect and the impact on the viability/morphology on cancer cells. It would also be of interest to study the effect of the chroman-4-ones in models of neurodegeneration.
- In the work regarding the PAL study, introduction of a ligation handle in the 2-position and the application of a bioorthogonal strategy should be investigated to facilitate the LC/MS-MS analysis. Studies to verify the incorporation of the probe into SIRT2 should also be addressed.
- Future work concerning the new cyclic sulfonamide scaffolds could include a series of derivatives lacking the polar NH- and carbonyl groups in the new scaffolds. Modelling studies for investigation of the increased SIRT3 activity of some of the new scaffold analogs could be starting point for a larger series of diverse derivatives as potential small-molecule SIRT3 inhibitors.

Nu är det dags att tacka ett flertal personer som på ett eller annat sätt har bidragit till den här avhandlingen. (Vem trodde för fem år sen att jag skulle komma att skriva detta på svenska!?)

Först och främst vill jag tacka min handledare, Prof. Kristina Luthman, för att du antog mig som doktorand. Tack för allt ditt stöd, dina uppmuntrande ord och allting du har lärt mig. Du är en fantastisk människa att se upp till.

Elina M. Jarho, min biträdande handledare för allting du lärde mig och för ditt stöd. Du är en så underbar person.

Mina examinatorer, Ann-Therese Karlberg och senare Johan Gottfries för givande samtal.

Maria Fridén-Saxin, du har varit med från dag ett på min tid på GU. Först som handledare på mitt ex-jobb, sen som en fantastisk kollega och nu som vän. Tack för alla trevliga stunder tillsammans med dig och din fina familj.

Marcus Malo, för ett trevligt samarbete med sirtuinerna. Tack så mycket för hårt arbete och alla fina bilder.

Karin Engen and Johanna Steén, mina examensarbetare, för att ni har kämpat hårt med kromanoner och sulfonamider. Ni har bidragit mycket till den här avhandlingen. Tack också till Bahram Khajehali, som syntetiserade det bicykliska kinolonderivatet.

Anna S.-S., för att du alltid finns där, för din positiva attityd och för alla sköna kaffepauser hos Ines.

Thanks to Elina, Tarja Kokkola and Maija Lahtela-Kakkonen for a very valuable collaboration and for your nice hospitality during our visits in Finland. Thanks also to Tiina Suuronen and Sari Ukkonen for all help with the sirtuin inhibition assays. And Tarja for help with the cell test.

Carina Sihlbom and **Johan Lengqvist**, för ett trevligt samarbete i PAL projektet, för allting jag fick lära mig om massspektrometri och för ert tålamod med alla mina frågor.

Helena Carén and Patricia Ruiz Ontañón for biological testing. Patricia, you crazy Spanish girl, it was really nice to have met you.

Alla andra medförfattare på peken, Erik A. A. Wallén, Kristian Meinander och Marie Rydén Landergren.

Jesper Nilsson för hjälp med den fotokemiska karakteriseringen.

Former and present members of the MedChem group who have made these five years to an incredible time. Ett särskild tack till **Mariell** för trevligt sällskap på olika konferenser. Jag kommer aldrig att glömma babushkan och kon i Moskva ⁽²⁾

The people from floor 8 and 9 for providing a friendly, stimulating working environment and nice get-togethers like Christmas and crayfish parties. All thanks to all of you who have kept our instruments running - you did a great job. **Mate Erdelyi**, stort tack för din otroliga hjälpsamhet med tolkning av NMR-spektra men också med så mycket annat.

Morgongänget, Susanne, Itedale, Elaine, PM, Jonathan och PO. Tack för trevliga samtal över en kaffe innan arbetsdagen började.

Maria, Itedale och Nadja för att korrekturläsa avhandlingen. Marcus och Tarja, tack för alla tips och givande kommentarer.

Und an meine Familie. Besonders an Mama und Papa, für euer unendliches Vertrauen und eure Unterstützung. Danke, dass ihr immer an mich geglaubt habt. Ein großer Dank auch an Gundula und Reiks Familie für eure Unterstützung.

Last but not least, **Reik**, für dein Verständnis, deine unglaubliche Geduld und Unterstützung. Aber am meisten für deine Liebe.

- 1. Frye, R. A. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem. Biophys.* Res. Commun. 2000, 273, 793-798.
- 2. Ivy, J. M.; Hicks, J. B.; Klar, A. J. S. Map Positions of Yeast Genes Sir1, Sir3 and Sir4. *Genetics* 1985, 111, 735-744.
- 3. Rine, J.; Herskowitz, I. 4 Genes Responsible for a Position Effect on Expression from Hml and Hmr in Saccharomyces-Cerevisiae. *Genetics* **1987**, *116*, 9-22.
- 4. Strahl, B. D.; Allis, C. D. The language of covalent histone modifications. *Nature* 2000, 403, 41-45.
- 5. Kouzarides, T. Acetylation: a regulatory modification to rival phosphorylation? *EMBO J.* **2000**, *19*, 1176-1179.
- 6. Polevoda, B.; Sherman, F. The diversity of acetylated proteins. *Genome Biology* **2002**, *3*.
- 7. Walsh, C. T.; Garneau-Tsodikova, S.; Gatto, G. J. Protein posttranslational modifications: The chemistry of proteome diversifications. *Angew. Chem. Int. Edit.* **2005**, *44*, 7342-7372.
- 8. Imai, S.; Armstrong, C. M.; Kaeberlein, M.; Guarente, L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* **2000**, *403*, 795-800.
- 9. De Ruijter, A. J. M.; Van Gennip, A. H.; Caron, H. N.; Kemp, S.; Van Kuilenburg, A. B. P. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem. J.* **2003**, *370*, 737-749.
- 10. Kaeberlein, M.; McVey, M.; Guarente, L. The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. *Genes Dev.* **1999**, *13*, 2570-2580.
- 11. Tissenbaum, H. A.; Guarente, L. Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. *Nature* **2001**, *410*, 227-230.
- 12. Lin, S. J.; Defossez, P. A.; Guarente, L. Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. *Science* **2000**, *289*, 2126-2128.
- 13. Guarente, L.; Picard, F. Calorie restriction the SIR2 connection. *Cell* 2005, *120*, 473-482.
- Colman, R. J.; Anderson, R. M.; Johnson, S. C.; Kastman, E. K.; Kosmatka, K. J.; Beasley, T. M.; Allison, D. B.; Cruzen, C.; Simmons, H. A.; Kemnitz, J. W.; Weindruch, R. Caloric Restriction Delays Disease Onset and Mortality in Rhesus Monkeys. *Science* 2009, *325*, 201-204.
- 15. Bordone, L.; Guarente, L. Calorie restriction, SIRT1 and metabolism: Understanding longevity. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 298-305.
- 16. Kanfi, Y.; Naiman, S.; Amir, G.; Peshti, V.; Zinman, G.; Nahum, L.; Bar-Joseph, Z.; Cohen, H. Y. The sirtuin SIRT6 regulates lifespan in male mice. *Nature* **2012**, *483*, 218-221.
- Mostoslavsky, R.; Chua, K. F.; Lombard, D. B.; Pang, W. W.; Fischer, M. R.; Gellon, L.; Liu, P. F.; Mostoslavsky, G.; Franco, S.; Murphy, M. M.; Mills, K. D.; Patel, P.; Hsu, J. T.; Hong, A. L.; Ford, E.; Cheng, H. L.; Kennedy, C.; Nunez, N.; Bronson, R.; Frendewey, D.; Auerbach, W.; Valenzuela, D.; Karow, M.; Hottiger, M. O.; Hursting, S.; Barrett, J. C.; Guarente, L.; Mulligan, R.; Demple, B.; Yancopoulos, G. D.; Alt, F. W. Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. *Cell* 2006, *124*, 315-329.
- 18. Bellizzi, D.; Rose, G.; Cavalcante, P.; Covello, G.; Dato, S.; De Rango, F.; Greco, V.; Maggiolini, M.; Feraco, E.; Mari, V.; Franceschi, C.; Passarino, G.; De Benedictis, G. A novel VNTR enhancer within the SIRT3 gene, a human homologue of SIR2, is associated with survival at oldest ages. *Genomics* **2005**, *85*, 258-263.
- 19. Heilbronn, L. K.; Ravussin, E. Calorie restriction and aging: review of the literature and implications for studies in humans. *Am. J. Clin. Nutr.* **2003**, *78*, 361-369.
- Burnett, C.; Valentini, S.; Cabreiro, F.; Goss, M.; Somogyvari, M.; Piper, M. D.; Hoddinott, M.; Sutphin, G. L.; Leko, V.; McElwee, J. J.; Vazquez-Manrique, R. P.; Orfila, A. M.; Ackerman, D.; Au, C.; Vinti, G.; Riesen, M.; Howard, K.; Neri, C.; Bedalov, A.; Kaeberlein, M.; Soti, C.; Partridge, L.; Gems, D. Absence of effects of Sir2 overexpression on lifespan in C. elegans and Drosophila. *Nature* 2011, 477, 482-U136.
- 21. Kaeberlein, M.; Powers, R. W. Sir2 and calorie restriction in yeast: A skeptical perspective. *Ageing Res. Rev* 2007, *6*, 128-140.
- 22. Frye, R. A. Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. *Biochem. Biophys. Res. Commun.* 1999, *260*, 273-279.

- 23. Haigis, M. C.; Guarente, L. P. Mammalian sirtuins emerging roles in physiology, aging, and calorie restriction. *Genes Dev.* 2006, *20*, 2913-2921.
- 24. Landry, J.; Sutton, A.; Tafrov, S. T.; Heller, R. C.; Stebbins, J.; Pillus, L.; Sternglanz, R. The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 5807-5811.
- 25. Nakagawa, T.; Guarente, L. Sirtuins at a glance. J. Cell Sci. 2011, 124, 833-838.
- Rauh, D.; Fischer, F.; Gertz, M.; Lakshminarasimhan, M.; Bergbrede, T.; Aladini, F.; Kambach, C.; Becker, C. F.; Zerweck, J.; Schutkowski, M.; Steegborn, C. An acetylome peptide microarray reveals specificities and deacetylation substrates for all human sirtuin isoforms. *Nat. Commun.* 2013, *4*, 2327.
- 27. Du, J. T.; Zhou, Y. Y.; Su, X. Y.; Yu, J. J.; Khan, S.; Jiang, H.; Kim, J.; Woo, J.; Kim, J. H.; Choi, B. H.; He, B.; Chen, W.; Zhang, S.; Cerione, R. A.; Auwerx, J.; Hao, Q.; Lin, H. N. Sirt5 Is a NAD-Dependent Protein Lysine Demalonylase and Desuccinylase. *Science* **2011**, *334*, 806-809.
- 28. Tan, M. J.; Peng, C.; Anderson, K. A.; Chhoy, P.; Xie, Z. Y.; Dai, L. Z.; Park, J.; Chen, Y.; Huang, H.; Zhang, Y.; Ro, J.; Wagner, G. R.; Green, M. F.; Madsen, A. S.; Schmiesing, J.; Peterson, B. S.; Xu, G. F.; Ilkayeva, O. R.; Muehlbauer, M. J.; Braulke, T.; Muhlhausen, C.; Backos, D. S.; Olsen, C. A.; McGuire, P. J.; Pletcher, S. D.; Lombard, D. B.; Hirschey, M. D.; Zhao, Y. M. Lysine Glutarylation Is a Protein Posttranslational Modification Regulated by SIRT5. *Cell Metab.* **2014**, *19*, 605-617.
- 29. Jiang, H.; Khan, S.; Wang, Y.; Charron, G.; He, B.; Sebastian, C.; Du, J. T.; Kim, R.; Ge, E.; Mostoslavsky, R.; Hang, H. C.; Hao, Q.; Lin, H. N. SIRT6 regulates TNF-alpha secretion through hydrolysis of long-chain fatty acyl lysine. *Nature* **2013**, *496*, 110-113.
- Ahuja, N.; Schwer, B.; Carobbio, S.; Waltregny, D.; North, B. J.; Castronovo, V.; Maechler, P.; Verdin, E. Regulation of insulin secretion by SIRT4, a mitochondrial ADP-ribosyltransferase. *J. Biol. Chem.* 2007, 282, 33583–33592.
- 31. Liszt, G.; Ford, E.; Kurtev, M.; Guarente, L. Mouse Sir2 homolog SIRT6 is a nuclear ADP-ribosyltransferase. J. Biol. Chem. 2005, 280, 21313-21320.
- 32. Sauve, A. A. Sirtuin chemical mechanisms. *Biochim. Biophys. Acta (BBA) Proteins Proteom.* 2010, 1804, 1591-1603.
- 33. Hawse, W. F.; Hoff, K. G.; Fatkins, D. G.; Daines, A.; Zubkova, O. V.; Schramm, V. L.; Zheng, W.; Wolberger, C. Structural insights into intermediate steps in the Sir2 deacetylation reaction. *Structure* **2008**, *16*, 1368-1377.
- Jin, L.; Wei, W. T.; Jiang, Y. B.; Peng, H.; Cai, J. H.; Mao, C.; Dai, H.; Choy, W.; Bemis, J. E.; Jirousek, M. R.; Milne, J. C.; Westphal, C. H.; Perni, R. B. Crystal Structures of Human SIRT3 Displaying Substrate-induced Conformational Changes. J. Biol. Chem. 2009, 284, 24394-24405.
- 35. Sauve, A. A.; Celic, I.; Avalos, J.; Deng, H. T.; Boeke, J. D.; Schramm, V. L. Chemistry of gene silencing: The mechanism of NAD(+)-dependent deacetylation reactions. *Biochemistry* **2001**, *40*, 15456-15463.
- 36. Smith, B. C.; Denu, J. M. Sir2 deacetylases exhibit nucleophilic participation of acetyl-lysine in NAD(+) cleavage. J. Am. Chem. Soc. 2007, 129, 5802-5803.
- Hu, P.; Wang, S. L.; Zhang, Y. K. Highly Dissociative and Concerted Mechanism for the Nicotinamide Cleavage Reaction in Sir2Tm Enzyme Suggested by Ab Initio QM/MM Molecular Dynamics Simulations. J. Am. Chem. Soc. 2008, 130, 16721-16728.
- 38. Smith, B. C.; Denu, J. M. Sir2 protein deacetylases: Evidence for chemical intermediates and functions of a conserved histidine. *Biochemistry* **2006**, *45*, 272-282.
- 39. Avalos, J. L.; Bever, K. M.; Wolberger, C. Mechanism of sirtuin inhibition by nicotinamide: Altering the NAD(+) cosubstrate specificity of a Sir2 enzyme. *Mol. Cell* **2005**, *17*, 855-868.
- 40. www.uniprot.org.
- 41. Flick, F.; Luscher, B. Regulation of sirtuin function by posttranslational modifications. *Front. Pharmacol.* **2012**, *3*, 29.
- 42. North, B. J.; Verdin, E. Interphase Nucleo-Cytoplasmic Shuttling and Localization of SIRT2 during Mitosis. *PLoS One* **2007**, *2*.
- Finnin, M. S.; Donigian, J. R.; Pavletich, N. P. Structure of the histone deacetylase SIRT2. *Nat. Struct. Biol.* 2001, *8*, 621-625.
- 44. Min, J. R.; Landry, J.; Sternglanz, R.; Xu, R. M. Crystal structure of a SIR2 homolog-NAD complex. *Cell* **2001**, *105*, 269-279.
- 45. Sanders, B. D.; Jackson, B.; Marmorstein, R. Structural basis for sirtuin function: What we know and what we don't. *Biochim. Biophys. Acta (BBA) Proteins Proteom.* **2010**, *1804*, 1604-1616.
- 46. Bellamacina, C. R. Protein motifs .9. The nicotinamide dinucleotide binding motif: A comparison of nucleotide binding proteins. *FASEB J.* **1996**, *10*, 1257-1269.

- 47. Avalos, J. L.; Celic, I.; Muhammad, S.; Cosgrove, M. S.; Boeke, J. D.; Wolberger, C. Structure of a Sir2 enzyme bound to an acetylated p53 peptide. *Mol. Cell* **2002**, *10*, 523-535.
- 48. Hoff, K. G.; Avalos, J. L.; Sens, K.; Wolberger, C. Insights into the sirtuin mechanism from ternary complexes containing NAD(+) and acetylated peptide. *Structure* **2006**, *14*, 1231-1240.
- 49. Avalos, J. L.; Boeke, J. D.; Wolberger, C. Structural basis for the mechanism and regulation of Sir2 enzymes. *Mol. Cell* **2004**, *13*, 639-648.
- 50. Moniot, S.; Schutkowski, M.; Steegborn, C. Crystal structure analysis of human Sirt2 and its ADP-ribose complex. *J. Struct. Biol.* **2013**, *182*, 136-143.
- 51. Cen, Y.; Youn, D. Y.; Sauve, A. A. Advances in Characterization of Human Sirtuin Isoforms: Chemistries, Targets and Therapeutic Applications. *Curr. Med. Chem.* 2011, *18*, 1919-1935.
- 52. Lavu, S.; Boss, O.; Elliott, P. J.; Lambert, P. D. Sirtuins novel therapeutic targets to treat age-associated diseases. *Nat. Rev. Drug Discovery* **2008**, *7*, 841-853.
- 53. Morris, B. J. Seven sirtuins for seven deadly diseases of aging. Free Radical Biol. Med. 2013, 56, 133-171.
- 54. Haigis, M. C.; Sinclair, D. A. Mammalian Sirtuins: Biological Insights and Disease Relevance. *Annu. Rev. Pathol. Mech.* **2010**, *5*, 253-295.
- 55. Someya, S.; Yu, W.; Hallows, W. C.; Xu, J.; Vann, J. M.; Leeuwenburgh, C.; Tanokura, M.; Denu, J. M.; Prolla, T. A. Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. *Cell* **2010**, *143*, 802-812.
- 56. Jeong, S. M.; Xiao, C. Y.; Finley, L. W. S.; Lahusen, T.; Souza, A. L.; Pierce, K.; Li, Y. H.; Wang, X. X.; Laurent, G.; German, N. J.; Xu, X. L.; Li, C. L.; Wang, R. H.; Lee, J.; Csibi, A.; Cerione, R.; Blenis, J.; Clish, C. B.; Kimmelman, A.; Deng, C. X.; Haigis, M. C. SIRT4 Has Tumor-Suppressive Activity and Regulates the Cellular Metabolic Response to DNA Damage by Inhibiting Mitochondrial Glutamine Metabolism. *Cancer Cell* **2013**, *23*, 450-463.
- 57. Nakagawa, T.; Lomb, D. J.; Haigis, M. C.; Guarente, L. SIRT5 Deacetylates Carbamoyl Phosphate Synthetase 1 and Regulates the Urea Cycle. *Cell* **2009**, *137*, 560-570.
- Mostoslavsky, R.; Chua, K. F.; Lombard, D. B.; Pang, W. W.; Fischer, M. R.; Gellon, L.; Liu, P.; Mostoslavsky, G.; Franco, S.; Murphy, M. M.; Mills, K. D.; Patel, P.; Hsu, J. T.; Hong, A. L.; Ford, E.; Cheng, H. L.; Kennedy, C.; Nunez, N.; Bronson, R.; Frendewey, D.; Auerbach, W.; Valenzuela, D.; Karow, M.; Hottiger, M. O.; Hursting, S.; Barrett, J. C.; Guarente, L.; Mulligan, R.; Demple, B.; Yancopoulos, G. D.; Alt, F. W. Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. *Cell* 2006, *124*, 315-329.
- 59. Roth, M.; Chen, W. Y. Sorting out functions of sirtuins in cancer. Oncogene 2014, 33, 1609-1620.
- 60. Mao, Z. Y.; Hine, C.; Tian, X.; Van Meter, M.; Au, M.; Vaidya, A.; Seluanov, A.; Gorbunova, V. SIRT6 Promotes DNA Repair Under Stress by Activating PARP1. *Science* **2011**, *332*, 1443-1446.
- Barber, M. F.; Michishita-Kioi, E.; Xi, Y. X.; Tasselli, L.; Kioi, M.; Moqtaderi, Z.; Tennen, R. I.; Paredes, S.; Young, N. L.; Chen, K. F.; Struhl, K.; Garcia, B. A.; Gozani, O.; Li, W.; Chua, K. F. SIRT7 links H3K18 deacetylation to maintenance of oncogenic transformation. *Nature* 2012, 487, 114-118.
- 62. North, B. J.; Marshall, B. L.; Borra, M. T.; Denu, J. M.; Verdin, E. The human Sir2 ortholog, SIRT2, is an NAD(+)-dependent tubulin deacetylase. *Mol. Cell* **2003**, *11*, 437-444.
- 63. Dryden, S. C.; Nahhas, F. A.; Nowak, J. E.; Goustin, A. S.; Tainsky, M. A. Role for human SIRT2 NADdependent deacetylase activity in control of mitotic exit in the cell cycle. *Mol. Cell. Biol.* 2003, *23*, 3173-3185.
- 64. Inoue, T.; Hiratsuka, M.; Osaki, M.; Oshimura, M. The molecular biology of mammalian SIRT proteins SIRT2 in cell cycle regulation. *Cell Cycle* **2007**, *6*, 1011-1018.
- 65. Vaquero, A.; Scher, M. B.; Lee, D. H.; Sutton, A.; Cheng, H. L.; Alt, F. W.; Serrano, L.; Sternglanz, R.; Reinberg, D. SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis. *Genes Dev.* **2006**, *20*, 1256-1261.
- 66. de Oliveira, R. M.; Sarkander, J.; Kazantsev, A. G.; Outeiro, T. F. SIRT2 as a Therapeutic Target for Age-Related Disorders. *Front. Pharmacol.* **2012**, *3*, 82.
- 67. Donmez, G.; Outeiro, T. F. SIRT1 and SIRT2: emerging targets in neurodegeneration. *Embo Molecular Medicine* **2013**, *5*, 344-352.
- 68. Hiratsuka, M.; Inoue, T.; Toda, T.; Kimura, N.; Shirayoshi, Y.; Kamitani, H.; Watanabe, T.; Ohama, E.; Tahimic, C. G. T.; Kurimasa, A.; Oshimura, M. Proteomics-based identification of differentially expressed genes in human gliomas: down-regulation of SIRT2 gene. *Biochem. Biophys. Res. Commun.* 2003, 309, 558-566.

- Kim, H. S.; Vassilopoulos, A.; Wang, R. H.; Lahusen, T.; Xiao, Z.; Xu, X.; Li, C.; Veenstra, T. D.; Li, B.; Yu, H.; Ji, J.; Wang, X. W.; Park, S. H.; Cha, Y. I.; Gius, D.; Deng, C. X. SIRT2 maintains genome integrity and suppresses tumorigenesis through regulating APC/C activity. *Cancer Cell* 2011, *20*, 487-499.
- 70. He, X.; Nie, H.; Hong, Y. Y.; Sheng, C. B.; Xia, W. L.; Ying, W. H. SIRT2 activity is required for the survival of C6 glioma cells. *Biochem. Biophys. Res. Commun.* **2012**, *417*, 468-472.
- 71. Xie, H. J.; Jung, K. H.; Nam, S. W. Overexpression of SIRT2 contributes tumor cell growth in hepatocellular carcinomas. *Mol. Cell. Toxicol.* 2011, 7, 367-374.
- Liu, P. Y.; Xu, N.; Malyukova, A.; Scarlett, C. J.; Sun, Y. T.; Zhang, X. D.; Ling, D.; Su, S. P.; Nelson, C.; Chang, D. K.; Koach, J.; Tee, A. E.; Haber, M.; Norris, M. D.; Toon, C.; Rooman, I.; Xue, C.; Cheung, B. B.; Kumar, S.; Marshall, G. M.; Biankin, A. V.; Liu, T. The histone deacetylase SIRT2 stabilizes Myc oncoproteins. *Cell Death Differ.* 2013, *20*, 503-514.
- 73. Li, Y. Z.; Matsumori, H.; Nakayama, Y.; Osaki, M.; Kojima, H.; Kurimasa, A.; Ito, H.; Mori, S.; Katoh, M.; Oshimura, M.; Inoue, T. SIRT2 down-regulation in HeLa can induce p53 accumulation via p38 MAPK activation-dependent p300 decrease, eventually leading to apoptosis. *Genes Cells* 2011, *16*, 34-45.
- 74. Di Fruscia, P.; Ho, K. K.; Laohasinnarong, S.; Khongkow, M.; Kroll, S. H. B.; Islam, S. A.; Sternberg, M. J. E.; Schmidtkunz, K.; Jung, M.; Lam, E. W. F.; Fuchter, M. J. The discovery of novel 10,11-dihydro-5H-dibenz[b,f]azepine SIRT2 inhibitors. *MedChemComm* 2012, *3*, 373-378.
- 75. Hoffmann, G.; Breitenbuecher, F.; Schuler, M.; Ehrenhofer-Murray, A. E. A novel SIRT2 inhibitor with p53-dependent pro-apoptotic activity in non-small-cell lung cancer. *J. Biol. Chem.* **2013**.
- Maxwell, M. M.; Tomkinson, E. M.; Nobles, J.; Wizeman, J. W.; Amore, A. M.; Quinti, L.; Chopra, V.; Hersch, S. M.; Kazantsev, A. G. The Sirtuin 2 microtubule deacetylase is an abundant neuronal protein that accumulates in the aging CNS. *Hum. Mol. Genet.* 2011, 20, 3986-3996.
- 77. Outeiro, T. F.; Kontopoulos, E.; Altmann, S. M.; Kufareva, I.; Strathearn, K. E.; Amore, A. M.; Volk, C. B.; Maxwell, M. M.; Rochet, J. C.; McLean, P. J.; Young, A. B.; Abagyan, R.; Feany, M. B.; Hyman, B. T.; Kazantsev, A. G. Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease. *Science* 2007, *317*, 516-519.
- Dawson, T. M.; Dawson, V. L. Molecular pathways of neurodegeneration in Parkinson's disease. *Science* 2003, 302, 819-822.
- 79. Esteves, A. R.; Arduino, D. M.; Swerdlow, R. H.; Oliveira, C. R.; Cardoso, S. M. Microtubule depolymerization potentiates alpha-synuclein oligomerization. *Front. Aging Neurosci.* 2010, 1.
- Luthi-Carter, R.; Taylor, D. M.; Pallos, J.; Lambert, E.; Amore, A.; Parker, A.; Moffitt, H.; Smith, D. L.; Runne, H.; Gokce, O.; Kuhn, A.; Xiang, Z. M.; Maxwell, M. M.; Reeves, S. A.; Bates, G. P.; Neri, C.; Thompson, L. M.; Marsh, J. L.; Kazantsev, A. G. SIRT2 inhibition achieves neuroprotection by decreasing sterol biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* 2010, *107*, 7927-7932.
- Taylor, D. M.; Balabadra, U.; Xiang, Z. M.; Woodman, B.; Meade, S.; Amore, A.; Maxwell, M. M.; Reeves, S.; Bates, G. P.; Luthi-Carter, R.; Lowden, P. A. S.; Kazantsev, A. G. A Brain-Permeable Small Molecule Reduces Neuronal Cholesterol by Inhibiting Activity of Sirtuin 2 Deacetylase. *ACS Chem. Biol.* 2011, *6*, 540-546.
- Chopra, V.; Quinti, L.; Kim, J.; Vollor, L.; Narayanan, K. L.; Edgerly, C.; Cipicchio, P. M.; Lauver, M. A.; Choi, S. H.; Silverman, R. B.; Ferrante, R. J.; Hersch, S.; Kazantsev, A. G. The Sirtuin 2 Inhibitor AK-7 Is Neuroprotective in Huntington's Disease Mouse Models. *Cell Reports* 2012, *2*, 1492-1497.
- 83. Bobrowska, A.; Donmez, G.; Weiss, A.; Guarente, L.; Bates, G. SIRT2 Ablation Has No Effect on Tubulin Acetylation in Brain, Cholesterol Biosynthesis or the Progression of Huntington's Disease Phenotypes In Vivo. *PLoS One* 2012, *7*.
- Grozinger, C. M.; Chao, E. D.; Blackwell, H. E.; Moazed, D.; Schreiber, S. L. Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. *J. Biol. Chem.* 2001, 276, 38837-38843.
- 85. Cen, Y. Sirtuins inhibitors: The approach to affinity and selectivity. *Biochim. Biophys. Acta* 2010, 1804, 1635-1644.
- Heltweg, B.; Gatbonton, T.; Schuler, A. D.; Posakony, J.; Li, H. Z.; Goehle, S.; Kollipara, R.; DePinho, R. A.; Gu, Y. S.; Simon, J. A.; Bedalov, A. Antitumor activity of a small-molecule inhibitor of human silent information regulator 2 enzymes. *Cancer Res.* 2006, *66*, 4368-4377.
- Medda, F.; Russell, R. J. M.; Higgins, M.; McCarthy, A. R.; Campbell, J.; Slawin, A. M. Z.; Lane, D. P.; Lain, S.; Westwood, N. J. Novel Cambinol Analogs as Sirtuin Inhibitors: Synthesis, Biological Evaluation, and Rationalization of Activity. *J. Med. Chem.* 2009, *52*, 2673-2682.

- Medda, F.; Joseph, T. L.; Pirrie, L.; Higgins, M.; Slawin, A. M. Z.; Lain, S.; Verma, C.; Westwood, N. J. N1-Benzyl substituted cambinol analogues as isozyme selective inhibitors of the sirtuin family of protein deacetylases. *MedChemComm* 2011, 2, 611-615.
- Mahajan, S. S.; Scian, M.; Sripathy, S.; Posakony, J.; Lao, U.; Loe, T. K.; Leko, V.; Thalhofer, A.; Schuler, A. D.; Bedalov, A.; Simon, J. A. Development of Pyrazolone and Isoxazol-5-one Cambinol Analogues as Sirtuin Inhibitors. J. Med. Chem. 2014, 57, 3283-3294.
- Suzuki, T.; Khan, M. N. A.; Sawada, H.; Imai, E.; Itoh, Y.; Yamatsuta, K.; Tokuda, N.; Takeuchi, J.; Seko, T.; Nakagawa, H.; Miyata, N. Design, Synthesis, and Biological Activity of a Novel Series of Human Sirtuin-2-Selective Inhibitors. *J. Med. Chem.* 2012, *55*, 5760-5773.
- 91. Suzuki, T.; Imai, K.; Nakagawa, H.; Miyata, N. 2-anilinobenzamides as SIRT inhibitors. *ChemMedChem* 2006, 1, 1059-1062.
- Trapp, J.; Jochum, A.; Meier, R.; Saunders, L.; Marshall, B.; Kunick, C.; Verdin, E.; Goekjian, P.; Sippl, W.; Jung, M. Adenosine mimetics as inhibitors of NAD(+)-dependent histone deacetylases, from kinase to sirtuin inhibition. *J. Med. Chem.* 2006, *49*, 7307-7316.
- 93. Napper, A. D.; Hixon, J.; McDonagh, T.; Keavey, K.; Pons, J. F.; Barker, J.; Yau, W. T.; Amouzegh, P.; Flegg, A.; Hamelin, E.; Thomas, R. J.; Kates, M.; Jones, S.; Navia, M. A.; Saunders, J.; DiStefano, P. S.; Curtis, R. Discovery of indoles as potent and selective inhibitors of the deacetylase SIRT1. *J. Med. Chem.* 2005, 48, 8045-8054.
- 94. Lain, S.; Hollick, J. J.; Campbell, J.; Staples, O. D.; Higgins, M.; Aoubala, M.; McCarthy, A.; Appleyard, V.; Murray, K. E.; Baker, L.; Thompson, A.; Mathers, J.; Holland, S. J.; Stark, M. J. R.; Pass, G.; Woods, J.; Lane, D. P.; Westwood, N. J. Discovery, in vivo activity, and mechanism of action of a small-molecule p53 activator. *Cancer Cell* **2008**, *13*, 454-463.
- 95. Neugebauer, R. C.; Uchiechowska, U.; Meier, R.; Hruby, H.; Valkov, V.; Verdin, E.; Sippl, W.; Jung, M. Structure-activity studies on splitomicin derivatives as sirtuin inhibitors and computational prediction of binding mode. *J. Med. Chem.* **2008**, *51*, 1203-1213.
- Kiviranta, P. H.; Suuronen, T.; Wallen, E. A. A.; Leppanen, J.; Tervonen, J.; Kyrylenko, S.; Salminen, A.; Poso, A.; Jarho, E. M. N-epsilon-Thioacetyl-Lysine-Containing Tri-, Tetra-, and Pentapeptides as SIRT1 and SIRT2 Inhibitors. J. Med. Chem. 2009, 52, 2153-2156.
- Mellini, P.; Kokkola, T.; Suuronen, T.; Salo, H. S.; Tolvanen, L.; Mai, A.; Lahtela-Kakkonen, M.; Jarho, E. M. Screen of Pseudopeptidic Inhibitors of Human Sirtuins 1-3: Two Lead Compounds with Antiproliferative Effects in Cancer Cells. J. Med. Chem. 2013, 56, 6681-6695.
- Maurer, B.; Rumpf, T.; Scharfe, M.; Stolfa, D. A.; Schmitt, M. L.; He, W. J.; Verdin, E.; Sippl, W.; Jung, M. Inhibitors of the NAD(+)-Dependent Protein Desuccinylase and Demalonylase Sirt5. ACS Med. Chem. Lett. 2012, 3, 1050-1053.
- Parenti, M. D.; Grozio, A.; Bauer, I.; Galeno, L.; Damonte, P.; Millo, E.; Sociali, G.; Franceschi, C.; Ballestrero, A.; Bruzzone, S.; Del Rio, A.; Nencioni, A. Discovery of Novel and Selective SIRT6 Inhibitors. J. Med. Chem. 2014, 57, 4796-4804.
- 100. He, B.; Du, J. T.; Lin, H. N. Thiosuccinyl Peptides as Sirt5-Specific Inhibitors. J. Am. Chem. Soc. 2012, 134, 1922-1925.
- 101. He, B.; Hu, J.; Zhang, X.; Lin, H. Thiomyristoyl peptides as cell-permeable Sirt6 inhibitors. Org. Biomol. Chem. 2014, 12, 7498-7502.
- 102. Howitz, K. T.; Bitterman, K. J.; Cohen, H. Y.; Lamming, D. W.; Lavu, S.; Wood, J. G.; Zipkin, R. E.; Chung, P.; Kisielewski, A.; Zhang, L. L.; Scherer, B.; Sinclair, D. A. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature* **2003**, *425*, 191-196.
- 103. Dai, H.; Kustigian, L.; Carney, D.; Case, A.; Considine, T.; Hubbard, B. P.; Perni, R. B.; Riera, T. V.; Szczepankiewicz, B.; Vlasuk, G. P.; Stein, R. L. SIRT1 activation by small molecules: kinetic and biophysical evidence for direct interaction of enzyme and activator. *J. Biol. Chem.* 2010, 285, 32695-32703.
- 104. Dittenhafer-Reed, K. E.; Feldman, J. L.; Denu, J. M. Catalysis and Mechanistic Insights into Sirtuin Activation. *ChemBioChem* 2011, 12, 281-289.
- Fridén-Saxin, M.; Pemberton, N.; Andersson, K. D.; Dyrager, C.; Friberg, A.; Grøtli, M.; Luthman, K. Synthesis of 2-Alkyl-Substituted Chromone Derivatives Using Microwave Irradiation. *J. Org. Chem.* 2009, 74, 2755-2759.
- 106. Dahlen, K.; Grotli, M.; Luthman, K. A scaffold approach to 3,6,8-trisubstituted flavones. *Synlett* 2006, 897-900.
- 107. Wallen, E. A. A.; Dahlen, K.; Grotli, M.; Luthman, K. Synthesis of 3-aminomethyl-2-aryl-8-bromo-6chlorochromones. Org. Lett. 2007, 9, 389-391.

- 108. Dyrager, C.; Nilsson Möllers, L.; Kjäll, L. K.; Alao, J. P.; Diner, P.; Wallner, F. K.; Sunnerhagen, P.; Grøtli, M. Design, Synthesis, and Biological Evaluation of Chromone-Based p38 MAP Kinase Inhibitors. *J. Med. Chem.* 2011, 54, 7427-7431.
- 109. Saengchantara, S. T.; Wallace, T. W. Chromanols, Chromanones, and Chromones. *Nat. Prod. Rep.* **1986**, *3*, 465-475.
- 110. Gaspar, A.; Matos, M. J.; Garrido, J.; Uriarte, E.; Borges, F. Chromone: A Valid Scaffold in Medicinal Chemistry. *Chem. Rev.* 2014, *114*, 4960-4992.
- 111. Di Carlo, G.; Mascolo, N.; Izzo, A. A.; Capasso, F. Flavonoids: Old and new aspects of a class of natural therapeutic drugs. *Life Sci.* **1999**, *65*, 337-353.
- 112. Horton, D. A.; Bourne, G. T.; Smythe, M. L. The combinatorial synthesis of bicyclic privileged structures or privileged substructures. *Chem. Rev.* 2003, *103*, 893-930.
- 113. Evans, B. E.; Rittle, K. E.; Bock, M. G.; Dipardo, R. M.; Freidinger, R. M.; Whitter, W. L.; Lundell, G. F.; Veber, D. F.; Anderson, P. S.; Chang, R. S. L.; Lotti, V. J.; Cerino, D. J.; Chen, T. B.; Kling, P. J.; Kunkel, K. A.; Springer, J. P.; Hirshfield, J. Methods for Drug Discovery - Development of Potent, Selective, Orally Effective Cholecystokinin Antagonists. J. Med. Chem. 1988, 31, 2235-2246.
- 114. Inaba, T.; Tanaka, K.; Takeno, R.; Nagaki, H.; Yoshida, C.; Takano, S. Synthesis and antiinflammatory activity of 7-methanesulfonylamino-6-phenoxychromones. Antiarthritic effect of the 3-formylamino compound (T-614) in chronic inflammatory disease models. *Chem. Pharm. Bull.* **2000**, *48*, 131-139.
- 115. Albrecht, U.; Lalk, M.; Langer, P. Synthesis and structure-activity relationships of 2-vinylchroman-4-ones as potent antibiotic agents. *Bioorg. Med. Chem.* **2005**, *13*, 1531-1536.
- 116. Raj, T.; Bhatia, R. K.; Sharma, R. K.; Gupta, V.; Sharma, D.; Ishara, M. P. S. Mechanism of unusual formation of 3-(5-phenyl-3H-[1,2,4]dithiazol-3-yl)chromen-4-ones and 4-oxo-4H-chromene-3-carbothioic acid N-phenylamides and their antimicrobial evaluation. *Eur. J. Med. Chem.* **2009**, *44*, 3209-3216.
- 117. Zhou, T.; Shi, Q. A.; Chen, C. H.; Zhu, H.; Huang, L.; Ho, P.; Lee, K. H. Anti-AIDS agents 79. Design, synthesis, molecular modeling and structure-activity relationships of novel dicamphanoyl-2 ',2 '- dimethyldihydropyrano-chromone (DCP) analogs as potent anti-HIV agents. *Bioorg. Med. Chem.* 2010, *18*, 6678-6689.
- 118. Ragab, F. A.; Yahya, T. A. A.; El-Naa, M. M.; Arafa, R. K. Design, synthesis and structure-activity relationship of novel semi-synthetic flavonoids as antiproliferative agents. *Eur. J. Med. Chem.* 2014, *82*, 506-520.
- 119. Baker, W. Molecular rearrangement of some o-acyloxyacetophenones and the mechanism of the production of 3-acylchromones. J. Chem. Soc. 1933, 1381-1389.
- 120. Mahal, H. S.; Rai, H. S.; Venkataraman, K. Synthetical experiments in the chromone group Part XV A synthesis of formononetin, daidzein, and psi-baptigenin. J. Chem. Soc. 1934, 1769-1771.
- 121. Yoshida, M.; Fujino, Y.; Doi, T. Synthesis of gamma-Benzopyranone by TfOH-Promoted Regioselective Cyclization of o-Alkynoylphenols. Org. Lett. 2011, 13, 4526-4529.
- 122. Yoshida, M.; Fujino, Y.; Saito, K.; Doi, T. Regioselective synthesis of flavone derivatives via DMAP-catalyzed cyclization of o-alkynoylphenols. *Tetrahedron* **2011**, *67*, 9993-9997.
- 123. Awuah, E.; Capretta, A. Access to Flavones via a Microwave-Assisted, One-Pot Sonogashira-Carbonylation-Annulation Reaction. Org. Lett. 2009, 11, 3210-3213.
- 124. Ankner, T.; Fridén-Saxin, M.; Pemberton, N.; Seifert, T.; Grøtli, M.; Luthman, K.; Hilmersson, G. KHMDS Enhanced SmI₂-Mediated Reformatsky Type a-Cyanation. *Org. Lett.* **2010**, *12*, 2210-2213.
- 125. Kabbe, H. J.; Widdig, A. Synthesis and Reactions of 4-Chromanones. Angew. Chem. Int. Ed. 1982, 21, 247-256.
- 126. Biddle, M. M.; Lin, M.; Scheidt, K. A. Catalytic enantioselective synthesis of flavanones and chromanones. J. Am. Chem. Soc. 2007, 129, 3830-3831.
- 127. Wang, L. J.; Liu, X. H.; Dong, Z. H.; Fu, X.; Feng, X. M. Asymmetric Intramolecular Oxa-Michael Addition of Activated alpha,beta-Unsaturated Ketones Catalyzed by a Chiral N,N '-Dioxide Nickel(II) Complex: Highly Enantioselective Synthesis of Flavanones. *Angew. Chem. Int. Ed.* **2008**, *47*, 8670-8673.
- 128. Stubbing, L. A.; Li, F. F.; Furkert, D. P.; Caprio, V. E.; Brimble, M. A. Access to 2-alkyl chromanones via a conjugate addition approach. *Tetrahedron* **2012**, *68*, 6948-6956.
- 129. Vila, C.; Hornillos, V.; Fananas-Mastral, M.; Feringa, B. L. Catalytic asymmetric conjugate addition of Grignard reagents to chromones. *Chem. Commun. (Cambridge, U. K.)* **2013**, *49*, 5933-5935.
- 130. Freedman, T. B.; Cao, X. L.; Dukor, R. K.; Nafie, L. A. Absolute configuration determination of chiral molecules in the solution state using vibrational circular dichroism. *Chirality* **2003**, *15*, 743-758.
- 131. The computanial calculations were performed at AstraZeneca R&D, Mölndal, Sweden.

- 132. http://www.enzolifesciences.com/.
- 133. Wegener, D.; Wirsching, F.; Riester, D.; Schwienhorst, A. A fluorogenic histone deacetylase assay well suited for high-throughput activity screening. *Chem. Biol.* **2003**, *10*, 61-68.
- 134. McDougal, P. G.; Rico, J. G.; Oh, Y. I.; Condon, B. D. A Convenient Procedure for the Monosilylation of Symmetrical 1,*n*-Diols. J. Org. Chem. **1986**, *51*, 3388-3390.
- 135. Tojo, G.; Fernandez, M. Oxidation of Alcohols to Aldehydes and Ketones: A Guide to Current Common Practice. Oxidation of Alcohols to Aldehydes and Ketones: A Guide to Current Common Practice 2006, 1-375.
- 136. Shah, S. T. A.; Singh, S.; Guiry, P. J. A Novel, Chemoselective and Efficient Microwave-Assisted Deprotection of Silyl Ethers with Selectfluor. J. Org. Chem. 2009, 74, 2179-2182.
- 137. Paley, M. S.; Harris, J. M. Synthesis of the Aldehyde of Oligomeric Polyoxyethylene. *Journal of Polymer Science Part a-Polymer Chemistry* **1987**, *25*, 2447-2454.
- 138. Heravi, M. M.; Ajami, D.; Tabar-Heydar, K. Oxidation of alcohols by montmorillonite K-10 supported bis(trimethylsilyl)chromate. *Monatsh. Chem.* **1998**, *129*, 1305-1308.
- Effenberger, F.; Hartmann, W. Enoläther, VI. Synthesen mit β-Äthoxy-acrylsäurechloriden. *Chem. Ber.* 1969, 102, 3260-3267.
- 140. Tietze, L. F.; Schneider, C.; Pretor, M. Improved Synthesis of (E)-3-Alkoxy- and (E)-3-Phenoxyacryloyl Chlorides. *Synthesis-Stuttgart* **1993**, 1079-1080.
- 141. Zaragoza, F.; Stephensen, H.; Peschke, B.; Rimvall, K. 2-(4-alkylpiperazin-1-yl)quinolines as a new class of imidazole-free histamine H-3 receptor antagonists. *J. Med. Chem.* **2005**, *48*, 306-311.
- 142. Battistuzzi, G.; Cacchi, S.; Fabrizi, G. An efficient palladium-catalyzed synthesis of cinnamaldehydes from acrolein diethyl acetal and aryl iodides and bromides. *Org. Lett.* **2003**, *5*, 777-780.
- 143. Quinn, J. F.; Razzano, D. A.; Golden, K. C.; Gregg, B. T. 1,4-cyclohexadiene with Pd/C as a rapid, safe transfer hydrogenation system with microwave heating. *Tetrahedron Lett.* **2008**, *49*, 6137-6140.
- 144. Zhao, X.; Allison, D.; Condon, B.; Zhang, F. Y.; Gheyi, T.; Zhang, A. P.; Ashok, S.; Russell, M.; MacEwan, I.; Qian, Y. W.; Jamison, J. A.; Luz, J. G. The 2.5 Å Crystal Structure of the SIRT1 Catalytic Domain Bound to Nicotinamide Adenine Dinucleotide (NAD(+)) and an Indole (EX527 Analogue) Reveals a Novel Mechanism of Histone Deacetylase Inhibition. J. Med. Chem. 2013, 56, 963-969.
- 145. Yuan, H.; Marmorstein, R. Structural Basis for Sirtuin Activity and Inhibition. J. Biol. Chem. 2012, 287, 42428-42435.
- 146. Yamagata, K.; Goto, Y.; Nishimasu, H.; Morimoto, J.; Ishitani, R.; Dohmae, N.; Takeda, N.; Nagai, R.; Komuro, I.; Suga, H.; Nureki, O. Structural basis for potent inhibition of SIRT2 deacetylase by a macrocyclic peptide inducing dynamic structural change. *Structure* **2014**, *22*, 345-352.
- 147. Friesner, R. A.; Murphy, R. B.; Repasky, M. P.; Frye, L. L.; Greenwood, J. R.; Halgren, T. A.; Sanschagrin, P. C.; Mainz, D. T. Extra precision glide: Docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J. Med. Chem.* 2006, *49*, 6177-6196.
- 148. Wilcken, R.; Zimmermann, M. O.; Lange, A.; Joerger, A. C.; Boeckler, F. M. Principles and Applications of Halogen Bonding in Medicinal Chemistry and Chemical Biology. *J. Med. Chem.* **2013**, *56*, 1363-1388.
- 149. Peck, B.; Chen, C. Y.; Ho, K. K.; Di Fruscia, P.; Myatt, S. S.; Coombes, R. C.; Fuchter, M. J.; Hsiao, C. D.; Lam, E. W. F. SIRT Inhibitors Induce Cell Death and p53 Acetylation through Targeting Both SIRT1 and SIRT2. *Mol. Cancer Ther.* 2010, *9*, 844-855.
- McCarthy, A. R.; Sachweh, M. C. C.; Higgins, M.; Campbell, J.; Drummond, C. J.; van Leeuwen, I. M. M.; Pirrie, L.; Ladds, M. J. G. W.; Westwood, N. J.; Lain, S. Tenovin-D3, a Novel Small-Molecule Inhibitor of Sirtuin SirT2, Increases p21 (CDKN1A) Expression in a p53-Independent Manner. *Mol. Cancer Ther.* 2013, *12*, 352-360.
- 151. Cheon, M. G.; Kim, W.; Choi, M.; Kim, J.-E. AK-1, a specific SIRT2 inhibitor, induces cell cycle arrest by downregulating Snail in HCT116 human colon carcinoma cells. *Cancer Letters*. In press.
- 152. Singh, A.; Westheimer, F. H.; Thornton, E. R. Photolysis of Diazoacetylchymotrypsin. J. Biol. Chem. 1962, 237, 3006-3008.
- 153. Dubinsky, L.; Krom, B. P.; Meijler, M. M. Diazirine based photoaffinity labeling. *Bioorg. Med. Chem.* 2012, 20, 554-570.
- 154. Robinette, D.; Neamati, N.; Tomer, K. B.; Borchers, C. H. Photoaffinity labeling combined with mass spectrometric approaches as a tool for structural proteomics. *Expert Rev. Proteomics* **2006**, *3*, 399-408.
- 155. Geurink, P. P.; Prely, L. M.; van der Marel, G. A.; Bischoff, R.; Overkleeft, H. S. Photoaffinity Labeling in Activity-Based Protein Profiling. *Activity-Based Protein Profiling* **2012**, *324*, 85-113.
- Shields, C. J.; Chrisope, D. R.; Schuster, G. B.; Dixon, A. J.; Poliakoff, M.; Turner, J. J. Photochemistry of Aryl Azides - Detection and Characterization of a Dehydroazepine by Time-Resolved Infrared-Spectroscopy and Flash-Photolysis at Room-Temperature. J. Am. Chem. Soc. 1987, 109, 4723-4726.

- 157. Schnapp, K. A.; Poe, R.; Leyva, E.; Soundararajan, N.; Platz, M. S. Exploratory Photochemistry of Fluorinated Aryl Azides Implications for the Design of Photoaffinity-Labeling Reagents. *Bioconjugate Chem.* **1993**, *4*, 172-177.
- 158. Smith, R. A. G.; Knowles, J. R. Aryldiazirines Potential Reagents for Photolabeling of Biological Receptor Sites. J. Am. Chem. Soc. 1973, 95, 5072-5073.
- 159. Brunner, J.; Senn, H.; Richards, F. M. "3-Trifluoromethyl-3-Phenyldiazirine a New Carbene Generating Group for Photolabeling Reagents. J. Biol. Chem. 1980, 255, 3313-3318.
- 160. Hashimoto, M.; Hatanaka, Y. Recent progress in diazirine-based photoaffinity labeling. *Eur. J. Org. Chem.* **2008**, 2513-2523.
- 161. Sadaghiani, A. M.; Verhelst, S. H. L.; Bogyo, M. Tagging and detection strategies for activity-based proteomics. *Curr. Opin. Chem. Biol.* 2007, *11*, 20-28.
- 162. Lapinsky, D. J. Tandem photoaffinity labeling-bioorthogonal conjugation in medicinal chemistry. *Bioorg. Med. Chem.* **2012**, *20*, 6237-6247.
- 163. Saxon, E.; Bertozzi, C. R. Cell surface engineering by a modified Staudinger reaction. *Science* **2000**, *287*, 2007-2010.
- 164. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. A stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem. Int. Ed.* **2002**, *41*, 2596-2599.
- 165. Steen, H.; Mann, M. The ABC's (and XYZ's) of peptide sequencing. *Nat. Rev. Mol. Cell Biol.* 2004, *5*, 699-711.
- 166. Yip, G. M. S.; Chen, Z. W.; Edge, C. J.; Smith, E. H.; Dickinson, R.; Hohenester, E.; Townsend, R. R.; Fuchs, K.; Sieghart, W.; Evers, A. S.; Franks, N. P. A propofol binding site on mammalian GABA(A) receptors identified by photolabeling. *Nat. Chem. Biol.* 2013, *9*, 715-720.
- 167. LeRiche, T.; Skorey, K.; Roy, P.; McKay, D.; Bateman, K. P. Using mass spectrometry to study the photo-affinity labeling of protein tyrosine phosphatase 1B. *Int. J. Mass Spectrom.* **2004**, *238*, 99-106.
- 168. He, B.; Velaparthi, S.; Pieffet, G.; Pennington, C.; Mahesh, A.; Holzle, D. L.; Brunsteiner, M.; van Breemen, R.; Blond, S. Y.; Petukhov, P. A. Binding Ensemble Profiling with Photoaffinity Labeling (BEProFL) Approach: Mapping the Binding Poses of HDAC8 Inhibitors. *J. Med. Chem.* 2009, *52*, 7003-7013.
- 169. Kalesh, K. A.; Tate, E. W. A succinyl lysine-based photo-cross-linking peptide probe for Sirtuin 5. Org. Biomol. Chem. 2014, 12, 4310-4313.
- 170. Li, G. Q.; Samadder, P.; Arthur, G.; Bittman, R. Synthesis and antiproliferative properties of a photoactivatable analogue of ET-18-OCH3. *Tetrahedron* **2001**, *57*, 8925-8932.
- 171. Barral, K.; Moorhouse, A. D.; Moses, J. E. Efficient conversion of aromatic amines into azides: A onepot synthesis of triazole linkages. *Org. Lett.* **2007**, *9*, 1809-1811.
- 172. Chee, G. L.; Yalowich, J. C.; Bodner, A.; Wu, X.; Hasinoff, B. B. A diazirine-based photoaffinity etoposide probe for labeling topoisomerase II. *Bioorg. Med. Chem.* **2010**, *18*, 830-838.
- 173. Brown, N.; Jacoby, E. On scaffolds and hopping in medicinal chemistry. *Mini-Rev. Med. Chem.* 2006, 6, 1217-1229.
- 174. Bohm, H. J.; Flohr, A.; Stahl, M. Scaffold hopping. Drug. Discov. Today Technol. 2004, 1, 217-224.
- 175. Andersson, M. I.; MacGowan, A. P. Development of the quinolones. J. Antimicrob. Chemother. 2003, 51, 1-11.
- 176. Taylor, R. M.; Topliss, J. G. Structure-Activity Relationships of 3-Substituted Dihydrobenzothiadiazine Diuretics. J. Med. Chem. 1962, 5, 312-320.
- 177. Battisti, U. M.; Jozwiak, K.; Cannazza, G.; Puia, G.; Stocca, G.; Braghiroli, D.; Parenti, C.; Brasili, L.; Carrozzo, M. M.; Citti, C.; Troisi, L. 5-Arylbenzothiadiazine Type Compounds as Positive Allosteric Modulators of AMPA/Kainate Receptors. ACS Med. Chem. Lett. 2012, 3, 25-29.
- 178. Yamada, K. A.; Tang, C. M. Benzothiadiazides Inhibit Rapid Glutamate-Receptor Desensitization and Enhance Glutamatergic Synaptic Currents. J. Neurosci. 1993, 13, 3904-3915.
- 179. Zhan, P.; Liu, X.; De Clercq, E. Recent advances in antiviral activity of benzo/heterothiadiazine dioxide derivatives. *Curr. Med. Chem.* **2008**, *15*, 1529-1540.
- Moeker, J.; Peat, T. S.; Bornaghi, L. F.; Vullo, D.; Supuran, C. T.; Poulsen, S. A. Cyclic Secondary Sulfonamides: Unusually Good Inhibitors of Cancer-Related Carbonic Anhydrase Enzymes. *J. Med. Chem.* 2014, *57*, 3522-3531.
- Berends, A. C.; Luiten, P. G. M.; Nyakas, C. A review of the neuroprotective properties of the 5-HT1A receptor agonist repinotan HCl (BAY x 3702) in ischemic stroke. CNS Drug Rev. 2005, 11, 379-402.

- 182. Braghiroli, D.; Puia, G.; Cannazza, G.; Tait, A.; Parenti, C.; Losi, G.; Baraldi, M. Synthesis of 3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide derivatives as potential allosteric modulators of AMPA/kainate receptors. *J. Med. Chem.* **2002**, *45*, 2355-2357.
- 183. Pirotte, B.; de Tullio, P.; Boverie, S.; Michaux, C.; Lebrun, P. Impact of the Nature of the Substituent at the 3-Position of 4H-1,2,4-Benzothiadiazine 1,1-Dioxides on Their Opening Activity toward ATP-Sensitive Potassium Channels. J. Med. Chem. 2011, 54, 3188-3199.
- 184. Xu, L. A.; Shu, H.; Liu, Y.; Zhang, S. H.; Trudell, M. L. Oxidative cyclization of N-alkyl-o-methylarenesulfonamides to biologically important saccharin derivatives. *Tetrahedron* **2006**, *62*, 7902-7910.
- 185. Kamogawa, H.; Yamamoto, S.; Nanasawa, M. Syntheses of Saccharin and Cyclamate Derivatives Bearing Polymerizable Vinyl Group. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 3824-3827.
- 186. Burri, K. F. Tilcotil Studies .2. [4 + 2] Additions with Isothiazol-3(2h)-One 1,1-Dioxide. *Helv. Chim. Acta* **1990**, *73*, 69-80.
- Blanchet, J.; Macklin, T.; Ang, P.; Metallinos, C.; Snieckus, V. Directed Ortho metalation-cross coupling strategies. N-cumyl arylsulfonamides. Facile deprotection and expedient route to 7- and 4,7-substituted saccharins. J. Org. Chem. 2007, 72, 3199-3206.
- Desai, R. C.; Hlasta, D. J.; Monsour, G.; Saindane, M. T. An Efficient Large-Scale Synthesis of 4-Isopropyl-6-Benzisothiazolone and 4-Isopropyl-6-Methoxybenzisothiazolone. J. Org. Chem. 1994, 59, 7161-7163.
- 189. Qin, J.; Rao, A.; Chen, X. A.; Zhu, X. H.; Liu, Z. D.; Huang, X. H.; Degrado, S.; Huang, Y.; Xiao, D.; Aslanian, R.; Cheewatrakoolpong, B.; Zhang, H. T.; Greenfeder, S.; Farley, C.; Cook, J.; Kurowski, S.; Li, Q.; van Heek, M.; Chintala, M.; Wang, G. F.; Hsieh, Y. S.; Li, F. B.; Palani, A. Discovery of a Potent Nicotinic Acid Receptor Agonist for the Treatment of Dyslipidemia. ACS Med. Chem. Lett 2011, 2, 171-176.
- 190. Pain, C.; Celanire, S.; Guillaumet, G.; Joseph, B. Synthesis of 5-substituted 2-(4-or 3-methoxyphenyl)-4(1H)-quinolones. *Tetrahedron* **2003**, *59*, 9627-9633.
- Neidlein, R.; Humburg, G. Heterocyclic 12-Pi-Systems and 14-Pi-Systems .5. Synthesis of 6,8-Dichloro-3h-Naphtho[1,8-Be]Thiophen-3-One Iso-Pi-Electronic to Phenalenone. *Justus Liebigs Ann. Chem.* 1977, 904-909.