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2-Anilinobenzamides as SIRT Inhibitors

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Yeast silent information regulator 2 (Sir2) proteins are responsible for the establishment, maintenance, and regulation of gene silencing at mating type loci, telomeres, and rDNA and they act in this capacity by changing chromatin into a transcriptionally inactive state.[1–5] Transcriptional silencing by Sir2 is linked to its deacetylation of the acetylated lysine residues in the N-terminal tails of the histones in chromatin.[6,7] Thus, human SIRT1–7, homologues of the yeast Sir2 proteins, are categorized as Class III histone deacetylases (HDACs).[8] However, the target of SIRT regulatory deacetylation is not limited to histones. For example, SIRT1 catalyzes the deacetylation of p53,[9–11] and SIRT2 deacetylates α-tubulin.[12] Although the functions of SIRTs have not yet been determined, they have been suggested to be associated with certain disease states such as cancer[13,14] and HIV infection.[15] Therefore, SIRT inhibitors are of interest not only as tools for elucidating in detail the biological functions of the enzyme, but can also be considered as potential therapeutic agents.[16]

In contrast to Class I and Class II HDACs, which are zinc-dependent deacetylases, deacetylation by Class III HDACs is dependent on NAD^+.[17,18] In the deacetylation reaction of SIRTs, NAD^+ is hydrolyzed to release nicotinamide and the acetyl group of the acetylated lysine substrate is transferred to cleaved NAD^+, generating *O*-acetyl-ADP ribose.[19,20] To date, several classes of Sir2 or SIRT inhibitors have been reported (Figure 1).[14,15,18,21–26] Among these, nicotinamide is a potent SIRT inhibitor and it has been proposed that it inhibits SIRTs by binding to a conserved pocket adjacent to the NAD^+ binding pocket, thereby blocking NAD^+ hydrolysis.[18,21] EX-527, a recently reported SIRT1-selective inhibitor, is thought to inhibit SIRT1 by occupying the nicotinamide binding pocket.[22] Another SIRT inhibitor, carba-NAD^+, which is a nonhydrolyzable NAD^+ analogue, has been reported to inhibit a Sir2 homologue (HST2) by competing with NAD^+.[18] Very recently, cambinol has been reported as a SIRT1 and SIRT2 inhibitor that is competitive with histone H4-peptide substrates.[14]

Previous reports regarding SIRT inhibitors[18,21,22] suggested the presence of small-molecule inhibitors in a chemical library enriched with the structural families of nicotinamide and benzamide, which were expected to inhibit SIRTs by occupying NAD^+- or nicotinamide-binding pockets. We therefore evaluated the SIRT1 inhibition activity of our in-house compound library comprised of nicotinamide and benzamide derivatives (Figure 2) at a concentration of 300 μM, and the strongest inhibition was observed with 2-anilinobenzamide **7** (Table 1).

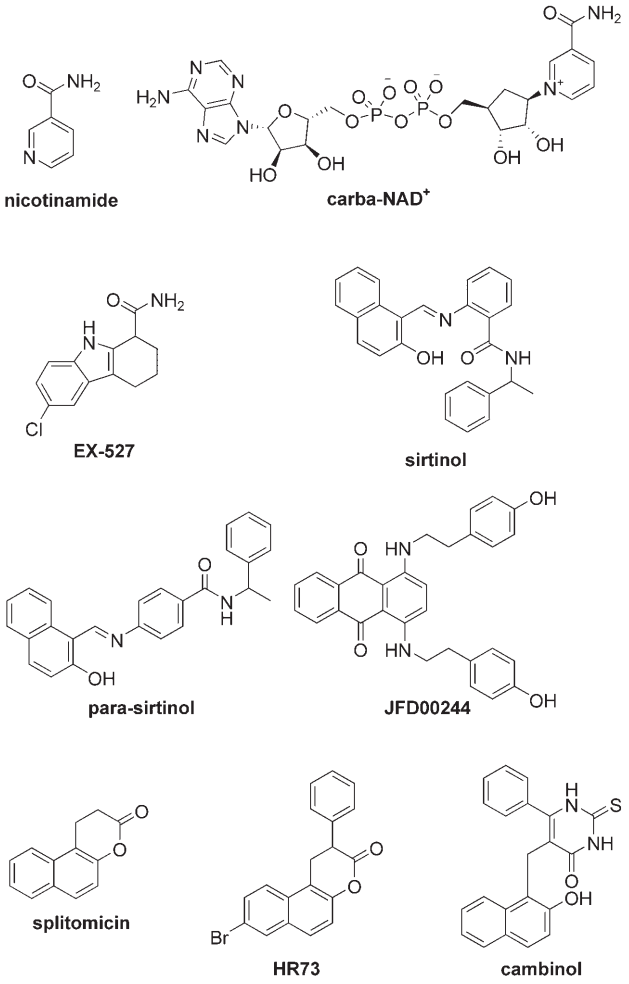

Figure 1. Sir2 and SIRT inhibitors.

To study the preliminary structure–activity relationship (SAR) of 2-anilinobenzamide derivatives, we prepared compounds **19**–**21** according to the route shown in Scheme 1. Carboxylic acid **22** was converted to *N*,*O*-dimethyl compound **23** by reac-

Table 1. SIRT1 inhibition data for compounds **1**–**18**[a]

Compd	Inhibition at 300 μM [%]	Compd	Inhibition at 300 μM [%]
1	0 ± 7.2	**10**	45 ± 1.1
2	72 ± 4.3	**11**	38 ± 2.4
3	4.6 ± 5.5	**12**	0 ± 1.6
4	42 ± 6.5	**13**	37 ± 9.6
5	52 ± 28	**14**	37 ± 2.8
6	42 ± 14	**15**	32 ± 5.4
7	100 ± 2.8	**16**	47 ± 6.1
8	8.4 ± 8.3	**17**	13 ± 9.3
9	47 ± 16	**18**	3.1 ± 9.7

[a] Values are means ± SD determined from at least three experiments.

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Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author. Supporting information includes experimental procedures and characterization data for compounds **19**, **20**, and **21**.

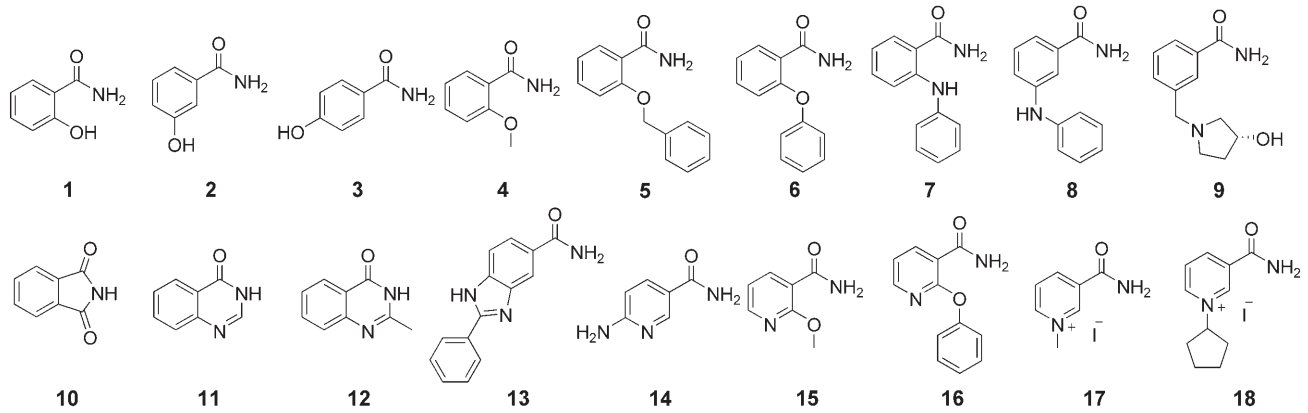
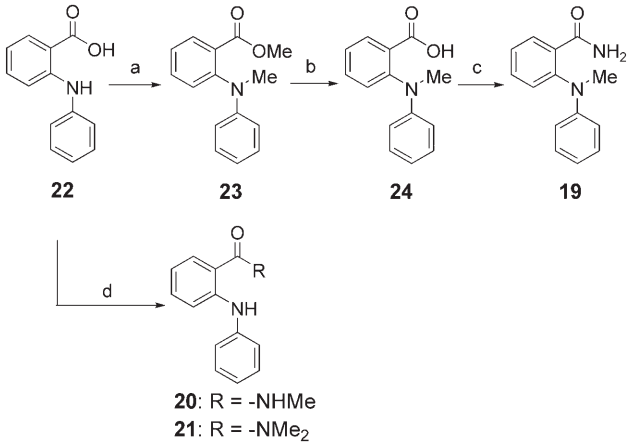


Figure 2. Examples of our benzamide- and nicotinamide-focused library.



Scheme 1. Reagents and conditions: a) 1) NaH, DMF, RT; 2) MeI, DMF, 80 °C, 99%; b) NaOH, MeOH, H_2O, RT, 99%; c) NH_4Cl, Et_3N, EDCI, HOBt, THF, RT, 69%; d) $MeNH_2$·HCl or Me_2NH·HCl, Et_3N, EDCI, HOBt, THF, RT, 60% for **20**, 37% for **21**.

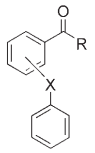
tion with methyl iodide in the presence of sodium hydride. Hydrolysis of the methyl ester of **23** gave *N*-methyl 2-anilinobenzoic acid **24**. Coupling between carboxylic acids **22**, **24**, and an appropriate amine afforded the desired benzamides **19**–**21**.

The results of the SIRT1 inhibition assay for compounds **6**–**8** and **19**–**22** are summarized in Table 2. Compound **7** exhibited an IC_{50} of 17 μM (see Figure S1 of Supporting Information) and its activity is comparable to that of nicotinamide. We initially tested the activity of compound **8**, the meta isomer of compound **7**, but it was found to be a much weaker inhibitor. We then examined the activity of compounds **6** and **19**, in which the NH group of compound **7** is replaced with an ether and NMe group, respectively. While ether **6** was totally inactive, *N*-methyl compound **19** slightly reduced potency. As for the conversion of the amide moiety, *N*-methyl amide **20** and *N*,*N*-dimethyl amide **21** significantly reduced the activity, whereas the potency of carboxylic acid **22** was maintained to some extent. The fact that carboxylic acid **22** displayed SIRT1 inhibitory activity was very surprising because the corresponding carboxylic acid derivatives of nicotinamide and EX-527 did not show any activity.[21,22] This indicated that compound **7** might inhibit SIRT1 in a manner different from nicotinamide and EX-527, although the structure of **7** and EX-527 is similar.

The unexpected SAR in the SIRT1 inhibition assay prompted us to investigate the SIRT1 inhibitory mechanism of compound **7**. We performed an enzyme kinetic assay (Lineweaver–Burk plot) using various concentrations of inhibitor **7** (Figure 3). Interestingly, the data from this study established that compound **7** engages in noncompetitive inhibition with NAD^+ and competitive inhibition with the acetylated lysine substrate.

Since compound **7** proved to be competitive with the acetylated lysine substrate and to act within the active site of SIRT1, the lowest energy conformation of **7** was obtained when it was docked into a model based on the crystal structure of yeast HST2 (PDB code 1Q1A),[20] a homologue of Sir2, as calculated using the software packages Glide 3.5 and MacroModel 8.1 (Figure 4). An inspection of the HST2/**7** complex suggests

Table 2. SIRT1 inhibition data for compounds **6**–**8** and **19**–**22**[a]



Compound	R	X	Position of XPh	Inhibition at 100 μM [%]	IC_{50} [μM]
nicotinamide				72 ± 11	25 ± 5.1
7	$-NH_2$	-NH-	*ortho*	81 ± 13	17 ± 1.8
6	$-NH_2$	-O-	*ortho*	4.3 ± 0.81	> 100
8	$-NH_2$	-NH-	*meta*	4.1 ± 1.4	> 100
19	$-NH_2$	-NMe-	*ortho*	62 ± 6.4	75 ± 5.6
20	-NHMe	-NH-	*ortho*	19 ± 1.2	> 100
21	$-NMe_2$	-NH-	*ortho*	39 ± 3.9	> 100
22	-OH	-NH-	*ortho*	52 ± 5.2	68 ± 2.9

[a] Values are means ± SD determined from at least three experiments.

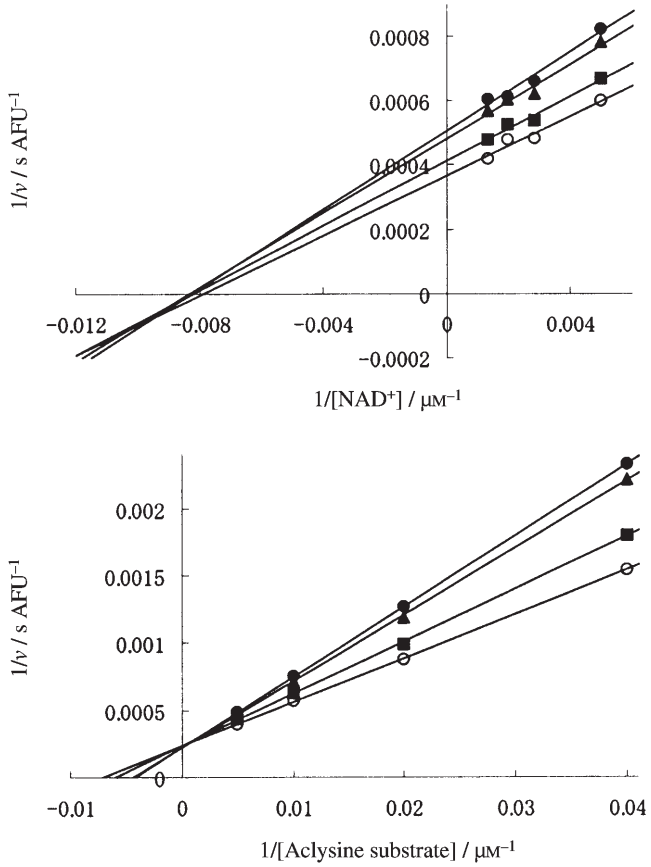


Figure 3. Reciprocal rate against reciprocal NAD^+ concentration (top) and acetylated lysine substrate (bottom) in the presence of 300 (●), 150 (▲), 50 (■), and 0 (○) μM of **7**.

that the NH_2 group and the CO group of **7** form hydrogen bonds with the backbone carbonyl of Ala 227 and with the backbone amine of Tyr 229, and the phenyl group of **7** blocks the entrance of the histone H4-binding pocket by interacting with hydrophobic amino acid residues (Val 182, Phe 184, Gly 185, Leu 188, Val 228, and Pro 238). In addition, an intramolecular hydrogen bond was observed between the CO and NH group of **7**. The results of the in vitro SAR and computational studies imply the importance of the conformation of the inhibitors. Specifically, conformation A of **7** is more stable than conformation B of **7** because of the intramolecular hydrogen bond between its CO and NH group (Figure 5). As the amide group of conformation A of **7** can form hydrogen bonds with the backbone amides of SIRT1, it would appear that compound **7** can strongly inhibit SIRT1. In contrast, conformation B of ether **6** is more stable than conformation A of **6** because of the intramolecular hydrogen bond between its NH group and oxygen atom. The amide group of conformation B of **6** cannot interact with the backbone amides of SIRT1, and this might be the reason that compound **6** lost SIRT1 inhibitory activity. In the case of *N*-methyl compound **19**, conformation A of **19** is more stable than conformation B of **19** due to steric repulsion of the NH group, pushing it away from the *N*-methyl group. Therefore, as with compound **7**, compound **19** can form hydrogen bonds with backbone amides of SIRT1 and this might be the reason that compound **19** showed a certain level of SIRT1-inhibitory activity.

To examine the isoform selectivity of compound **7**, we conducted enzyme assays using SIRT1, SIRT2, and SIRT3. Compound **7** showed about 4-fold and 14-fold selectivity for SIRT1 over SIRT 2 and SIRT3, respectively (IC_{50} for SIRT2 = 74 μM; IC_{50} for SIRT3 = 235 μM). In addition, compound **7** did not inhibit class I and class II HDACs at a concentration of 1000 μM.

To explore the potential for compound **7** to block SIRT1 activity in cells, we performed a cellular assay using western blot analysis. Since SIRT1 is known to catalyze the deacetylation of p53 on DNA damage,[9–11] the acetylation level of p53 in HCT116 cells after etoposide-induced DNA damage was analyzed.[27] As can be seen from Figure 6, elevated and dose-dependent levels of acetylated p53 were observed. These results suggested that compound **7** inhibits SIRT1 in cells.

In summary, to discover novel SIRT inhibitors, we evaluated a nicotinamide- and benzamide-focused chemical library to detect SIRT1 inhibition, and found 2-anilinobenzamide **7** to be a novel SIRT inhibitor. Although the structure of **7** is similar to that of EX-527, that of the SAR is not. The results of kinetic enzyme assays made it clear that compound **7** competes with the acetylated lysine substrate, whereas it has been reported that EX-527 does not.[21] Molecu-

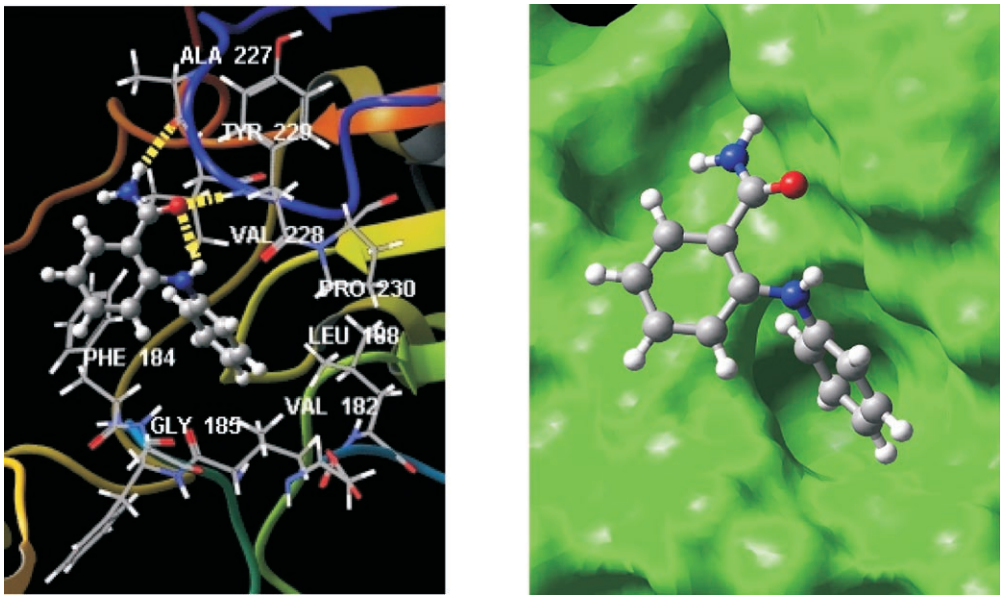


Figure 4. View of the conformation of **7** (ball and stick) docked into the yeast Hst2 (homologue of SIRTs) catalytic core. Residues 5 Å from **7** are displayed in the wire graphic (left) and the surface of the enzyme is displayed in the background (right).

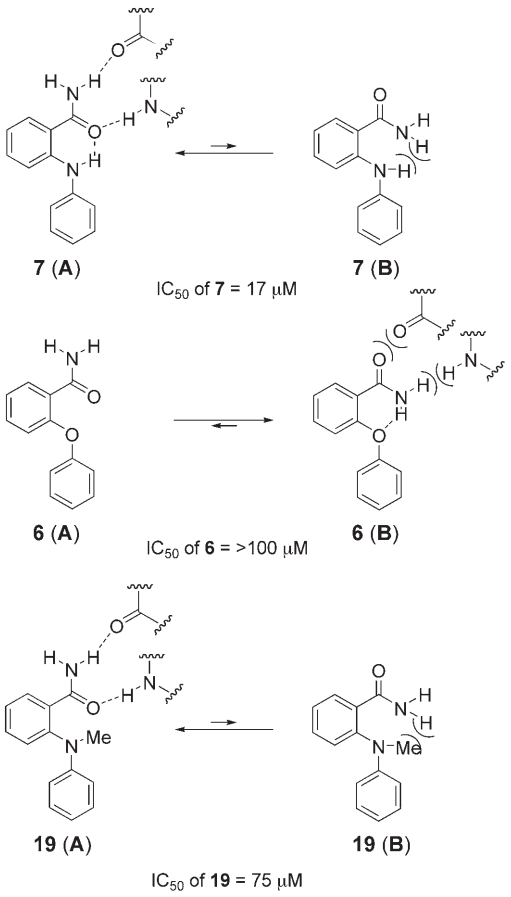


Figure 5. The relationship between SIRT1 inhibitory activity and the stable conformation of compounds **7**, **6**, and **19**.

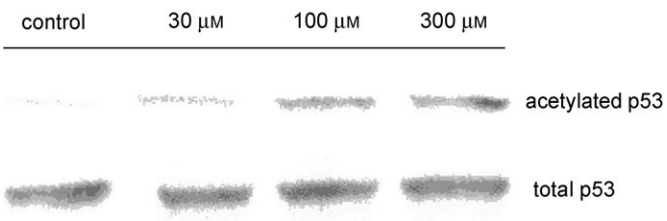


Figure 6. Western blot detection of acetylated p53 levels in HCT116 cells after an 8 h incubation with 20 μM of etoposide and various concentrations of compound **7**.

lar modeling suggests the significance of the conformation of inhibitors and the formation of hydrogen bonds between inhibitors and SIRTs. Compound **7** also caused p53 acetylation in cells, which would be the result of SIRT1 inhibition. These findings provide a basis for developing new tools for probing the biology of SIRTs and for finding new candidate therapeutic agents. Further investigations pertaining to **7** are progressing and will be reported in due course.

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Keywords: focused chemical library · kinetic analysis · p53 acetylation · SIRT inhibitors · structure–activity relationships

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[27] Etoposide was used to cause DNA damage and subsequent induction of p53. In the absence of etoposide, the induction of p53 was not observed (data not shown).

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