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Two-Step Synthesis of Novel, Bioactive Derivatives of the Ubiquitous Cofactor Nicotinamide Adenine Dinucleotide (NAD)

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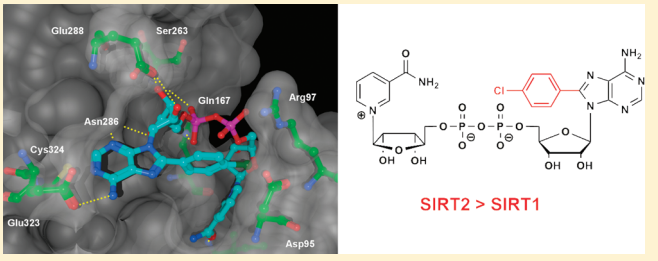
Supporting Information

ABSTRACT: We report the design and concise synthesis, in two steps from commercially available material, of novel, bioactive derivatives of the enzyme cofactor nicotinamide adenine dinucleotide (NAD). The new synthetic dinucleotides act as sirtuin (SIRT) inhibitors and show isoform selectivity for SIRT2 over SIRT1. An NMR-based conformational analysis suggests that the conformational preferences of individual analogues may contribute to their isoform selectivity.



INTRODUCTION

The dinucleotide NAD (nicotinamide adenine dinucleotide, Scheme 1, **1**) is a ubiquitous biomolecule with important functions in cellular energy metabolism and cell signaling.[1−3] Best known for its role as a redox cofactor, **1** is also an essential cosubstrate for a range of biologically important nonredox enzymes such as the poly(ADP ribose) polymerases (PARPs)[4,5] and sirtuins (SIRTs).[6,7] These enzymes require **1** for covalent modifications during group transfer reactions. The sirtuins, for example, catalyze the NAD-dependent deacetylation of acetyllysine residues in various protein substrates.[8] The seven members of the human sirtuin family (SIRT1−7) have been implicated in essential cellular processes including transcriptional control, cell cycle progression, and aging,[9] and individual sirtuins represent promising drug targets in, for example, cancer and diabetes.[10] Structural analogues of **1** are therefore sought as inhibitor candidates and chemical probes for the investigation of sirtuins and other NAD-dependent enzymes.[11]

Prompted by our interest in the development of sirtuin inhibitors, we have recently designed novel derivatives of **1** with an additional aromatic or heteroaromatic substituent in position 8 of the adenine base (Scheme 1). Molecular docking studies with the human sirtuin SIRT2[12] suggested that the NAD-binding site of this enzyme might be able to accommodate a sterically demanding substituent in this position (Figure 1). In order to test this hypothesis, we required efficient synthetic access to the target 8-(hetero)aryl-NAD derivatives. With a view toward analogue synthesis, we were particularly interested in a synthetic route that offered maximum structural flexibility. The only previous example for an 8-aryl-NAD derivative, 8-phenyl-NAD, has been prepared via a linear, multistep synthesis centered around the formation of the pyrophosphate bond.[13] Ideally, we wanted to avoid this synthetic step which can be plagued by variable yields,[14] focusing instead on the introduction of the 8-substituent on a preformed NAD scaffold. However, examples for the direct chemical modification of NAD are exceedingly rare and, because of the limited chemical stability of this biomolecule, generally require very mild conditions.

In principle, the target 8-(hetero)aryl substituted NAD derivatives are accessible by Suzuki−Miyaura cross-coupling[15] or direct arylation.[16−18] The direct structural modification of **1** is complicated by its water solubility, the presence of various unprotected functional groups, and several potential cleavage sites, including the particularly labile nicotinamide riboside bond. Previously reported conditions for the direct arylation of adenine nucleotides are therefore not compatible with the limited chemical stability of **1**.[16−18] While the Suzuki−Miyaura coupling of unprotected adenine nucleosides[19−22] and nucleotides, including adenosine triphosphate (ATP),[23] has been reported, no examples have been described to date for the application of this chemistry to an intact NAD substrate.

Herein, we report the successful implementation of this novel and highly efficient synthetic approach. We describe suitable conditions for the cross-coupling of 8-bromo-NAD **2**, including a one-pot/two-step procedure for the in situ bromination and cross-coupling of **1**. Results from biological assays with two sirtuin isoforms demonstrate that the target molecules act as inhibitors of SIRT2 but have generally only weak activity against SIRT1. A conformational analysis of the new NAD derivatives suggests that the conformational preferences of individual

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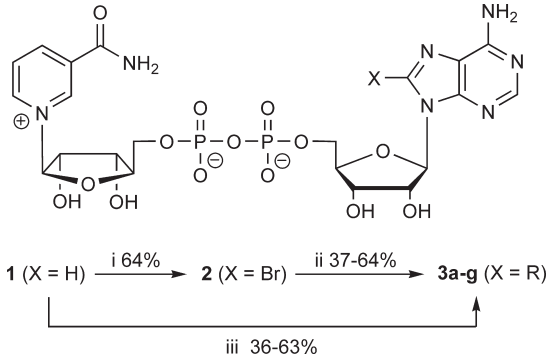
Scheme 1. Synthesis of 8-substituted NAD derivatives[a]



[a] Reagents and conditions i) aq. Br_2, aq. NaOAc (pH 4), rt, 1.75 h; ii) Na_2Cl_4Pd (2.5 mol %), TXPTS, R-B$(OH)_2$, K_2CO_3 (2 equiv), H_2O, 40 °C, 20−35 min; iii) neat. Br_2, aq. NaOAc (pH 4), rt, 0.5 h; then: Na_2Cl_4Pd (2.5 mol %), TXPTS, R-B$(OH)_2$, K_2CO_3 (5 equiv), H_2O, 40 °C, 20−30 min. For substituents R see Table 1.

analogues may contribute to their isoform selectivity. In principle, the new cofactor analogues may also be applicable as chemical probes for other NAD-dependent enzymes. Such applications will be greatly facilitated by our synthetic protocol, which provides an extremely short entry from commercially available material to this novel class of bioactive NAD derivatives.

RESULTS AND DISCUSSION

In order to develop a suitable Suzuki−Miyaura protocol, we first tested the hydrolytic stability of the potential cross-coupling substrate 8-bromo-NAD **2** (Scheme 1) under basic conditions. At pH 9, **2** was consumed completely within 15 min at 80 °C and within 60 min at 60 °C (Supporting Information). However, at 40 °C **2** proved to be remarkably stable for at least 1 h. We speculated that this stability may open a window of opportunity for the cross-coupling of **2**, provided the reaction could be carried out swiftly and efficiently. In order to exploit this opportunity, we set out to identify optimum cross-coupling conditions. Previously, we have successfully carried out cross-couplings of unprotected nucleotides and sugar nucleotides in water, using a catalytic system that combines the water-soluble ligand TPPTS (triphenylphosphine trisulfonate, sodium salt) and the Pd source Na_2Cl_4Pd.[24−26] However, in preliminary experiments with 8-bromoadenosine and phenylboronic acid, we found that these conditions necessitated elevated reaction temperatures not compatible with the limited stability of **2** (Supporting Information). In contrast, use of the electron-rich phosphine ligand TXPTS (tris(4,6-dimethyl-3-sulfonatophenyl)phosphine trisodium salt)[20] allowed the successful cross-coupling of 8-bromoadenosine at 40 °C within 1 h.

To apply these optimized cross-coupling conditions in the context of our synthetic strategy, 8-bromo-NAD **2** was required as the cross-coupling substrate. For the preparation of **2** we adapted a previously published protocol for the 8-selective bromination of NAD,[27] using saturated bromine water as the brominating agent instead of neat bromine (Scheme 1). After 1.75 h at room temperature, RP-HPLC indicated ∼90% conversion but also suggested the appearance of degradation products. The reaction was therefore stopped and the material purified by ion-pair chromatography, affording **2** in 64% isolated yield. With sufficient quantities of **2** in hand, we attempted the cross-coupling of **2** with a range of (hetero)arylboronic acids under our optimized

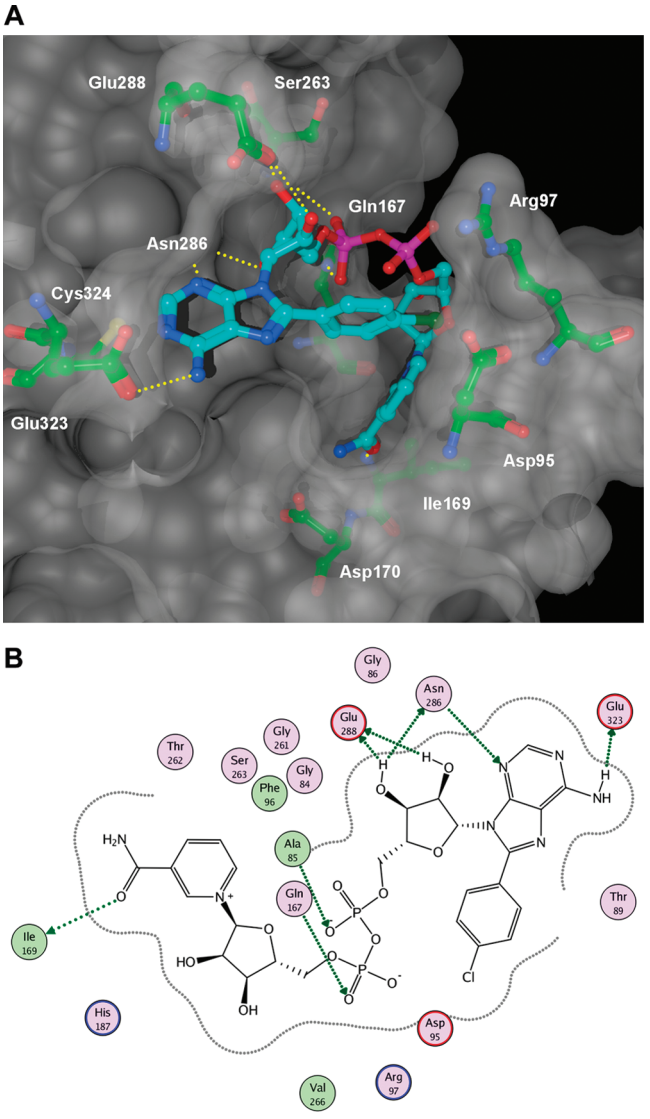


Figure 1. (A) Predicted docking pose for 8-(4-chlorophenyl)-NAD **3b** (cyan) in the cofactor binding site of human SIRT2 (ref 12). Hydrogen bonds are shown as yellow dashed lines. (B) Schematic representation of the **3b**−SIRT2 interactions. Hydrogen bonds are shown as green dashed lines.

Suzuki−Miyaura conditions (Scheme 1). Pleasingly, the cross-coupling of **2** was successful with both electron-rich and electron-poor phenylboronic acids, as well as furan-2-ylboronic acid (Table 1, entries 1−5). Because of the mild reaction conditions and short reaction times, cross-coupling products **3a**−**e** were generally obtained in moderate to good yields. Thus, this highly efficient approach allowed the preparation of a small library of 8-substituted NAD derivatives from a single synthetic precursor.

In order to further simplify analogue synthesis, we next investigated the combination of the bromination and cross-coupling steps in a one-pot, two-step procedure (Scheme 1). Using neat bromine instead of bromine−water, we were able to limit the bromination time to 30 min. While vigorous mixing was required for an effective dissolution of the bromine in the sodium acetate buffer, this protocol allowed us to suppress degradation of **1** or **2** during this step. Excess bromine was removed by simple extraction with $CHCl_3$. The aqueous solution was evaporated to dryness, and the crude residue was used in the subsequent cross-coupling step

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Table 1. Suzuki−Miyaura Coupling of 8-Bromo-NAD (2) with Various Aryl- and Heteroarylboronic Acids (R-B(OH)$_2$)

entry	compd	R	synthesis[a]	yield (%)[b]
1	**3a**	phenyl	i and ii	37
2	**3b**	4-chlorophenyl	i and ii	54
3	**3c**	4-methoxyphenyl	i and ii	58
4	**3d**	4-methylphenyl	i and ii	64
5	**3e**	furan-2-yl	i and ii	45
6	**3a**	phenyl	iii	63
7	**3f**	5-formylthien-2-yl	iii	36
8	**3g**	5-formylfuran-2-yl	iii	41

[a] See Scheme 1. [b] Isolated yields.

without further purification. As the bromination was carried out under slightly acidic conditions (pH 4), an additional 3 equiv of K_2CO_3 was required under these conditions to reach the optimum pH of 9 for the subsequent cross-coupling reaction.

The entire process, including both the bromination and cross-coupling steps, could be conveniently monitored by RP-HPLC (Figure S1, Supporting Information). This allowed a quick assessment of reaction progress and helped to avoid potential degradation of either starting materials or products. As expected, the cross-coupled products showed a significantly longer retention time than both **1** and **2**. The one-pot cross-coupling reaction was used successfully to prepare NAD derivatives **3a**, **3f**, and **3g** in moderate to good yields (Table 1, entries 6−8). It is noteworthy that the isolated yield of **3a** is comparable to the previously reported yield of 66% from multistep synthesis.[13] Advantageously, our protocol provides access to the target **3a** in a single operation and with short reaction times from commercially available **1**. This new approach therefore considerably facilitates the synthesis of novel and structurally diverse NAD derivatives on a milligram scale sufficient for biological experiments.

In order to assess the bioactivity of the novel 8-substituted NAD derivatives, compounds **2** and **3a**−**e** were evaluated as potential sirtuin inhibitors or cosubstrates. First, we carried out inhibition experiments with NAD derivatives **2** and **3a**−**e** and the human enzymes SIRT1 and SIRT2 (Table 2). Almost all test compounds, including the prototype NAD derivative **3a** bearing an 8-phenyl substituent, inhibited SIRT2 at low micromolar concentrations, while only moderate activity was observed against SIRT1. The presence of an additional group on the 8-phenyl substituent (**3b**−**d**) improved SIRT2 inhibition, while no such improvement was observed upon replacement of the phenyl with a furanyl ring (**3e**). IC_{50} values for the new 8-substituted NAD derivatives against SIRT2 were generally of a similar order of magnitude as the K_m for NAD previously reported for this enzyme[28] (Table 2). These results suggest that in general the additional substituent in position 8 is tolerated at the active site of human SIRT2, as predicted by molecular docking with the available X-ray structure[12] of this enzyme (Figure 1). At the same time, this structural modification appears to interfere with cofactor binding at SIRT1. 8-Substituted NAD derivatives may therefore provide a template for the development of isoform-selective sirtuin ligands. Since there is presently no X-ray structure available for SIRT1, we can only speculate about the structural basis for the differential effects the 8-substituent has on cofactor binding at the different sirtuin isoforms. A detailed analysis is complicated further by the relatively low sequence identity of 44% between SIRT1 and SIRT2. A sequence alignment of human SIRT1, -2, -3, and -5, indicating the differences in the NAD-binding region, has been included in the Supporting Information (Figure S2, Supporting Information).

Table 2. Sirtuin Inhibition Results[a]

compd	scaffold[b]	R	SIRT1[c]	SIRT2[d]
NAD	NAD	H	80 ± 9[e]	46 ± 12[e]
2	NAD	bromo	31%	28 ± 7
3a	NAD	phenyl	17%	123 ± 4
3b	NAD	4-chlorophenyl	30%	35 ± 8
3c	NAD	4-methoxyphenyl	18%	43 ± 5
3d	NAD	4-methylphenyl	20%	52 ± 12
3e	NAD	furan-2-yl	16%	35%[c]
4a	adenosine	bromo	inactive	16%[c]
4b	adenosine	phenyl	inactive	inactive[c]
5a	AMP	bromo	inactive	inactive[c]
5b	AMP	phenyl	31 ± 2[d]	29 ± 3

[a] K_m values for NAD are included for direct comparison. [b] See Scheme 1 and Figure 2. [c] Inhibition at 50 μM. Inactive: <10% inhibition. [d] IC_{50} ± SEM (μM). [e] K_m, from ref 28.

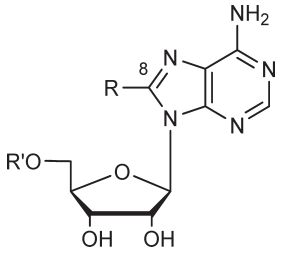

Figure 2. General structure of 8-substituted derivatives of adenosine (**4a** and **4b**, R′ = H) and AMP (**5a** and **5b**, R′ = PO_3^{2-}) (see Table 2).

In order to explore whether the complete dinucleotide scaffold is required for biological activity and/or isoform selectivity, we also tested the corresponding adenosine and adenosine monophosphate (AMP) congeners (Figure 2) of NAD derivatives **2** and **3a** against SIRT1 and SIRT2. The 8-substituted derivatives of adenosine (**4a**, **4b**) and AMP (**5a**, **5b**) were prepared as previously reported[13,22] (Supporting Information). The complete removal of the diphosphate and nicotinamide riboside in adenosine derivatives **4a** and **4b** led to a dramatic loss of activity. The mononucleotide 8-bromo-AMP **5a** was also inactive, while 8-phenyl-AMP **5b** was of a similar potency as the intact NAD derivatives. Importantly, however, and in contrast to the corresponding NAD derivative **3a**, **5b** displayed similar activity against both sirtuin subtypes. This suggests that in this series of sirtuin inhibitors, the complete NAD scaffold is crucial for enzyme selectivity.

It is a distinct possibility that, given their close structural similarity to NAD itself, the new 8-substituted derivatives may serve as cosubstrates for one or more members of the sirtuin family. Such a potential cosubstrate activity would have obvious implications for the interpretation of the observed inhibitory activity. We therefore also tested the new 8-substituted NAD derivatives for their reactivity as surrogate cosubstrates in the sirtuin-catalyzed conversion of an acetyllysine substrate. Under these conditions, only the 8-phenyl derivative **3a** led to a small conversion of the acetyllysine substrate when used as a substitute for NAD in this fluorescence-based assay (Figure 3). Importantly, however, even the conversion of **3a** is significantly lower

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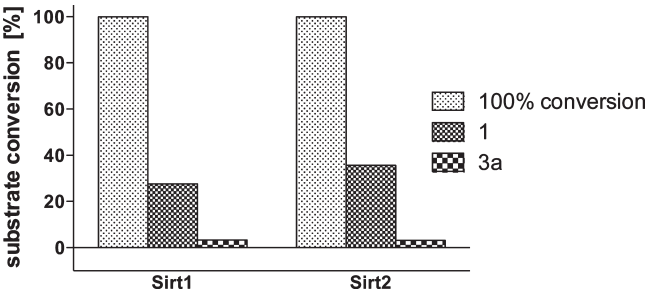


Figure 3. Substrate conversion with NAD (**1**) or 8-phenyl-NAD (**3a**) as cosubstrate. The 100% conversion refers to the signal obtained with 10.5 μM AMC (7-amino-4-methylcoumarin).

than that of NAD itself. These results suggest that none of the 8-substituted NAD derivatives is used productively as a cosubstrate by either SIRT1 or SIRT2.

To understand the structural basis for this lack of cosubstrate activity, we analyzed several available SIRT structures in the absence or presence of various ligands. No crystal structure has been reported to date for SIRT1, which made it impossible to study the interaction of the new NAD derivatives with the NAD binding site of SIRT1 by molecular modeling. However, it is known from the available crystal structures of human SIRT3 in its apo form and in complex with adenosine diphosphate ribose (ADPR) that a flexible loop in the NAD binding site adopts a completely different conformation in both structures.[29] This flexible loop contains NAD binding residues, and the relationship between its conformation and the presence of bound NAD is consistent among all SIRT homologues for which structures have been solved.[30] We therefore superimposed the X-ray structure of SIRT3 in complex with ADPR (PDB code 3GLT) with the structure of SIRT2 containing the docked inhibitor **3b** (Figure S3, Supporting Information). This superimposition indicates a steric clash between the 8-chlorophenyl substituent and the flexible binding region. Comparison with the SIRT3 X-ray structure in the apo form (PDB code 3GLS), on the other hand, shows the flexible loop in a conformation that can accommodate the 8-chlorophenyl substituent of **3b**. The conformation of the flexible loop is determined by the nature of the cosubstrate in the NAD binding pocket, and its movement has been implicated with SIRT catalysis and/or nicotinamide release.[30] On the basis of these overlays, it can be hypothesized that the 8-substituent in **2** and **3a**−**e** may interfere with this loop movement in SIRT2 and possibly in SIRT1, rendering these NAD derivatives poor sirtuin cosubstrates, as observed in our experiments.

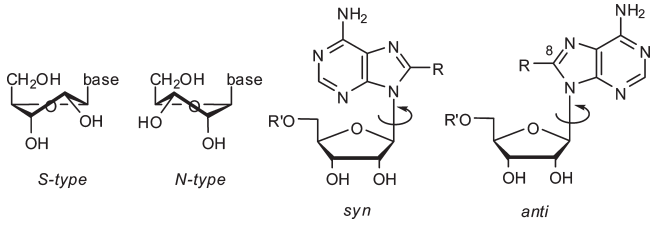
In order to gain a better understanding of preliminary structure−activity relationships (SAR) in this series, we carried out a conformational analysis of compounds **2** and **3a**−**e**. It is well-known that a substituent in position 8 can have a significant effect on the preferred conformation of NAD derivatives in solution.[31−33] Such conformational preferences may in turn have direct implications for the bioactivity of 8-substituted NAD derivatives. We were particularly interested in the conformation of the ribose ring in the adenosine fragment and in the orientation of the adenine base relative to the ribose. In solution, the ribose ring in nucleosides and nucleotides exists in an equilibrium between S-type (southern) conformations, which include the classical C(2′)-endo and C(3′)-exo conformers, and N-type (northern) conformations (Table 3).[31] The two main orientations of the adenine base are either toward the ribose (syn) or away from it (anti), depending on the glycosyl torsion.[32,33] Information about the preferred conformation of a given adenine nucleoside or nucleotide can be extracted from ^{1}H NMR data. Thus, the N-type/S-type ratio has been correlated experimentally with the magnitude of the H1′−H2′ coupling constant, and the relative percentage of the S-type conformer can be calculated as $10J_{1'2'}$.[31] The chemical shift of the H-2′ signal is a good indicator for the position of the syn/anti equilibrium.[32,33] In native NAD, which exists predominantly in the anti conformation, H-2′ resonates at 4.6 ppm. A downfield shift of this signal is generally indicative of a transition to the syn conformation.

Using this NMR-based approach, we have analyzed important conformational parameters of the 8-substituted NAD derivatives **2** and **3a**−**e** (Table 3). In solution, all analogues show a slight preference for the S-type conformation. Significantly, this is also the conformation of native, 8-unsubstituted NAD in the solved crystal structures with SIRT3,[29] SIRT5,[34] and bacterial enzymes.[30] The same NAD conformation might also be expected for the structurally related human SIRT2, which so far has been crystallized solely in the apo form.[12] This idea is supported by docking experiments with SIRT2 and the 8-substituted NAD analogues (Figure S4, Supporting Information). From the conformational analysis and the docking experiments it therefore appears that the additional substituent in position 8 does not perturb, and may indeed stabilize, the bioactive conformation of the adenosine ribose. For all 8-substituted analogues, we also observed a significant, albeit variable, downfield shift of the H-2′ signal relative to 8-unsubstituted NAD. This suggests that the 8-substituted derivatives adopt preferentially a syn conformation, which can be attributed to nonbonded repulsion between the 8-substituent and the ribose ring.[32,33]

The results from this conformational analysis raise the intriguing possibility that conformational preferences may contribute to the observed isoform selectivity in this series of NAD derivatives.[40] The available structures of SIRTs in complex with NAD[29,30,34] show the cofactor in the anti conformation,[12] and our docking experiments suggest a similar binding pose for the 8-substituted analogues. As all 8-substituted NAD derivatives adopt preferentially the syn conformation in solution, these cofactor analogues very likely have to undergo a conformational change prior to binding at a target sirtuin. The inhibitory activity of **3a**−**d** suggests that in principle both SIRT1 and SIRT2 can induce this conformational change but that SIRT2 is better equipped to do so than SIRT1. The differential ability of individual sirtuins to induce this conformational change may be linked to a nonconserved three-amino-acid motif in the flexible loop which is close to the 8-substituent of the ligand (Figures S5 and S6, Supporting Information). However, in the absence of structural data for SIRT1 this possibility remains speculative.

The general idea that the conformational preferences of the new 8-substituted sirtuin ligands underpin their isoform selectivity is further supported experimentally by the biological results for furan-2-yl derivative **3e**. **3e** shows the largest downfield shift for H-2′ and thus the strongest preference for the syn conformation. Locked in the syn conformation, **3e** is the weakest SIRT inhibitor in this series and the only analogue not to show preferential inhibition of SIRT2. It therefore appears that in this new series of NAD derivatives, the relative ease with which a given analogue can adopt the anti conformation may be directly correlated with its SIRT2 inhibitory activity. These results may therefore provide a template for the rational development of SIRT2-selective inhibitors.

Table 3. Conformational Analysis of 8-Substituted NAD Derivatives



compd	R	S-type conformer (%) ($10J_{1,2}$)	H-2′ (ppm)	ΔH-2′ (ppm)[a]	syn/anti
2	bromo	59	5.23	0.61	syn
3a	phenyl	59−60	5.19	0.57	syn
3b	4-chlorophenyl	58−61	5.19	0.57	syn
3c	4-methoxyphenyl	52−60	5.20	0.58	syn
3d	4-methylphenyl	56−60	5.17	0.55	syn
3e	furan-2-yl	52−56	5.36	0.74	syn

[a] ΔH-2′ values have been calculated as the difference between the chemical shift of H-2′ of the 8-substituted derivatives and H-2′ of NAD (4.62 ppm).

CONCLUSION

We have developed a synthetic protocol for the Suzuki−Miyaura coupling of an intact NAD substrate, which has provided access to a small library of novel, 8-substituted NAD derivatives with biological activity against the human sirtuin SIRT2. For isoform selectivity, the complete NAD scaffold is required, illustrating the usefulness of our efficient synthetic route toward such analogues. Our protocol will facilitate further analogue synthesis and allow the systematic exploration of SAR in this exciting series of novel cofactor analogues. The conformational analysis of the novel NAD derivatives suggests that conformational preferences may underpin their isoform selectivity. These findings may therefore provide a starting point for the rational design of further analogues with improved activity and isoform selectivity.

EXPERIMENTAL SECTION

General. All chemicals were obtained commercially and used as received unless stated otherwise. TXPTS was synthesized from tris(2,4-dimethylphenyl)phosphane[35] as described by Gulyas and co-workers.[36] 8-Bromoadenosine **4a**, 8-phenyladenosine **4b**, 8-bromo-AMP **5a**, and 8-phenyl-AMP **5b** were prepared, with minor modifications, as previously reported.[13,22] TLC was performed on precoated aluminum plates (silica gel 60 F_{254}, Merck). Compounds were visualized by exposure to UV light (254 and 365 nm). All test compounds were characterized analytically by NMR (^{1}H, ^{13}C, and where appropriate ^{31}P), high-resolution mass spectrometry (HRMS), and HPLC and met the required purity criteria (>95% by HPLC). NMR spectra were recorded at 298 K on a Varian VXR 400 S spectrometer at 400 MHz (^{1}H) or on a Bruker Avance DPX-300 spectrometer at 300 MHz (^{1}H). Chemical shifts (δ) are reported in ppm and referenced to methanol (δ_H 3.34, δ_C 49.50) for solutions in D_2O. Coupling constants (J) are reported in Hz. Resonance allocations were made with the aid of COSY and HSQC experiments. Accurate electrospray ionization mass spectra (HR ESI-MS) were obtained on a Finnigan MAT 900 XLT mass spectrometer at the EPSRC National Mass Spectrometry Service Centre, Swansea, U.K. Preparative chromatography was performed on a Biologic LP chromatography system equipped with a peristaltic pump and a 254 nm UV optics module. Analytical chromatography (HPLC) was carried out on an Agilent 1200 machine equipped with a Supelcosil LC-18T column (25 cm × 4.6 mm, particle size 5 μm), a diode array detector (detection wavelengths of 254 and 280 nm), and a column oven (temperature of 25 °C). For details of the chromatographic conditions, see Supporting Information.

General Synthetic Method 1: Suzuki−Miyaura Cross-Coupling of 8-Br-NAD (2). A round-bottom flask was charged with 8-Br-NAD **2** (1 equiv), Na_2Cl_4Pd (0.1 equiv), TXPTS (0.25 equiv), K_2CO_3 (1.5 equiv), and arylboronic acid (1.2 equiv) and purged with N_2 for 15 min. Degassed H_2O (2 mL) was added to the reaction vessel, and the reaction mixture was heated at 40 °C for 25 min. Reaction progress was monitored by RP-HPLC. Upon completion of the reaction, the reaction mixture was filtered through a syringe filter (pore size of 0.45 μm). The filtrate was concentrated under reduced pressure, and the residual crude was purified sequentially by ion-pair and ion-exchange chromatography (purification methods 2 and 3; see Supporting Information).

General Synthetic Method 2: "One-Pot" Bromination and Suzuki−Miyaura Coupling of NAD. To a solution of **1** (1 equiv) in aqueous sodium acetate buffer (0.5 M, pH 4, 670 μL), neat bromine (16 μL, 10 equiv) was added dropwise under vigorous stirring. The mixture was stirred at room temperature for 30 min. The excess bromine was extracted with $CHCl_3$ (3×), and the aqueous solution was evaporated to dryness in vacuo. To the residual pale orange film were added Na_2Cl_4Pd (0.1 equiv), TXPTS (0.25 equiv), K_2CO_3 (5.0 equiv), and phenylboronic acid (1.2 equiv). The reaction vessel was purged with N_2 for 15 min. Degassed H_2O was added under a N_2 atmosphere, and the mixture (pH 9) was heated at 40 °C for the given time. Reaction progress was monitored by RP-HPLC. Upon completion of the reaction, the reaction mixture was filtered through a syringe filter (pore size of 0.45 μm). The filtrate was concentrated under reduced pressure, and the residual crude was purified sequentially by ion-pair and ion-exchange chromatography (purification methods 2 and 3; see Supporting Information).

8-Bromo-NAD (2)[13]. To a solution of NAD (60 mg, 90.4 μmol) in aqueous sodium acetate (0.5 M, 2 mL, pH 4), saturated aqueous bromine (1.7 mL) was added dropwise. The reaction mixture was stirred at room temperature for 1.75 h. The excess bromine was extracted with chloroform (3×). The aqueous solution was reduced in vacuo to give a pale orange film which was purified by ion-pair chromatography (purification method 1). The triethylammonium salt of the title compound was obtained as a glassy solid in 64% yield (42.8 mg, 57.6 μmol). HPLC: 4.52 min (98%). δ_H (400 MHz, D_2O) 4.18−4.48 (8H, m, H-3′, H-3″,

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H-4′, H-4″, H-5′, H-5″), 4.60 (1H, m, H-2″), 5.23 (1H, t, J = 5.9 Hz, H-2′), 5.95−5.99 (2H, m, H-1′, H-1″), 7.91 (1H, s, H-2), 8.21 (1H, dd, J = 5.6 and 8.5 Hz, Hn_5), 8.82 (1H, d, J = 8.1 Hz, Hn_4), 9.11 (1H, d, J = 6.4 Hz, Hn_6), 9.29 (s, 1H, Hn_2); δ_C (75.5 MHz, D_2O) 65.7 (C-5′), 66.4 (C-5″), 70.2 (C-3″), 71.5 (C-2″), 71.7 (C-3′), 78.5 (C-2′), 84.2 (C-4″), 87.9 (C-4′), 90.0 (C-1′), 100.9 (C-1″), 119.8 (C-5), 128.9 (Cn_5), 129.8 (C-8), 134.7 (Cn_3), 140.7 (Cn_2), 143.5 (Cn_6), 146.8 (Cn_4), 151.1 (C-4), 153.9 (C-2), 155.0 (C-6), 166.2 ($CONH_3$); δ_P (121 MHz, D_2O) −11.7 (d, $J_{P,P}$ = 20.6 Hz), −11.3 (d, $J_{P,P}$ = 20.6 Hz). m/z (ESI) 740.0138 $[M - H]^-$, $C_{21}H_{25}{}^{79}BrN_7O_{14}P_2$ requires 740.0124.

8-Phenyl-NAD (3a)[13]**.** *From* ***2***. The triethylammonium (TEA) salt of the title compound was obtained as a glassy solid from **2** (17.0 mg, 22.9 μmol) and phenylboronic acid in 37% yield (7.0 mg, 0.8 equiv of TEA) according to general synthetic method 1 (reaction time, 25 min).

From ***1***. The triethylammonium (TEA) salt of the title compound was obtained as a glassy solid from **1** (20.0 mg, 30.1 μmol) and phenylboronic acid in 63% yield (16.6 mg, 1.0 equiv of TEA) according to general synthetic method 2 (cross-coupling time, 30 min). HPLC: 10.90 min (96%). δ_H (400 MHz, D_2O) 4.12−4.42 (8H, m, H-3′, H-3″, H-4′, H-4″, H-5′, H-5″), 4.44 (1H, t, J = 5.6 Hz, H-2″), 5.19 (1H, t, J = 6.0 Hz, H-2′), 5.83 (1H, d, J = 5.9 Hz, H-1′), 5.94 (1H, d, J = 5.2 Hz, H-1″), 7.59−7.68 (5H, m, Ph), 8.17 (1H, t, J = 7.6 Hz, Hn_5), 8.22 (1H, s, H-2), 8.72 (1H, d, J = 8.0 Hz, Hn_4), 9.10 (1H, d, J = 6.1 Hz, Hn_6), 9.25 (1H, s, Hn_2); δ_C (125 MHz, D_2O) 65.5, 66.4, 70.0, 71.0, 71.1, 78.3, 83.6, 87.6, 89.7, 100.7, 118.9, 128.2, 129.5, 129.8, 130.1, 131.9, 134.2, 140.5, 143.0, 146.3, 150.6, 152.9, 153.6, 155.4, 165.6; δ_P (121 MHz, D_2O) −11.7 (d, $J_{P,P}$ = 20.6 Hz), −11.3 (d, $J_{P,P}$ = 20.6 Hz). m/z (ESI) 738.1328, $[M - 2H]^-$, $C_{27}H_{30}N_7O_{14}P_2$ requires 738.1331.

8-(4-Chlorophenyl)-NAD (3b). The triethylammonium (TEA) salt of the title compound was obtained as a glassy solid from **2** (19.4 mg, 26.1 μmol) and 4-chlorophenylboronic acid in 54% yield (13.7 mg, 1.2 equiv of TEA) according to general synthetic method 1 (reaction time, 35 min). HPLC: 14.72 min (98%). δ_H (400 MHz, D_2O) 4.12−4.40 (8H, m, H-3′, H-3″, H-4′, H-4″, H-5′, H-5″), 4.48 (1H, t, J = 5.5 Hz, H-2″), 5.19 (1H, t, J = 6.1 Hz, H-2′), 5.78 (1H, d, J = 5.8 Hz, H-1′), 5.94 (1H, d, J = 5.2 Hz, H-1″), 7.50 (2H, d, J = 8.0 Hz, Ph), 7.56 (2H, d, J = 8.4 Hz, Ph), 8.18 (1H, t, J = 7.2 Hz, Hn_5), 8.19 (1H, s, H-2), 8.74 (1H, d, J = 8.0 Hz, Hn_4), 9.10 (1H, d, J = 6.3 Hz, Hn_6), 9.25 (1H, s, Hn_2); δ_C (125 MHz, D_2O) 65.4, 66.3, 70.0, 70.9, 71.1, 78.2, 83.5, 87.6, 89.6, 100.7, 118.7, 126.4, 129.4, 129.8, 131.0, 134.2, 137.5, 140.5, 143.0, 146.3, 150.5, 151.9, 153.1, 155.3, 165.6; δ_P (121 MHz, D_2O) −11.8 (d, $J_{P,P}$ = 20.6 Hz), −11.3 (d, $J_{P,P}$ = 20.6 Hz). m/z (ESI) 772.0952, $[M - H]^-$, $C_{27}H_{29}Cl^{35}N_7O_{14}P_2$ requires 772.0942.

8-(4-Methoxyphenyl)-NAD (3c). The triethylammonium (TEA) salt of the title compound was obtained as a glassy solid from **2** (17.0 mg, 22.9 μmol) and 4-methoxyphenylboronic acid in 58% yield (11.7 mg, 1.0 equiv of TEA) according to general synthetic method 1 (reaction time, 20 min). HPLC: 12.93 min (98%). δ_H (400 MHz, D_2O) 3.86 (3H, s, MeO), 4.12−4.40 (8H, m, H-3′, H-3″, H-4′, H-4″, H-5′, H-5″), 4.49 (1H, t, J = 5.8 Hz, H-2″), 5.20 (1H, t, J = 6 Hz, H-2′), 5.78 (1H, d, J = 5.2 Hz, H-1′), 5.90 (1H, d, J = 5.2 Hz, H-1″), 6.99 (2H, d, J = 8.8 Hz, *m*Ph), 7.49 (2H, d, J = 9.2 Hz, *o*Ph), 8.158 (1H, s, H-2), 8.162 (1H, t, J = 7.2 Hz, Hn_5), 8.72 (1H, d, J = 8.1 Hz, Hn_4), 9.08 (1H, d, J = 6.2 Hz, Hn_6), 9.22 (1H, s, Hn_2); δ_C (125 MHz, D_2O) 56.1, 65.4, 66.3, 70.0, 70.9, 71.1, 78.2 (C-2′), 83.4, 87.6, 89.7, 100.7, 115.0, 118.8, 120.4, 129.4, 131.4, 134.1, 140.4, 143.0, 146.3, 150.6, 152.4, 153.1, 155.0, 161.6, 165.6; δ_P (121 MHz, D_2O) −11.8 (d, $J_{P,P}$ = 20.6 Hz), −11.3 (d, $J_{P,P}$ = 20.6 Hz). m/z (ESI) 768.1426, $[M - 2H]^-$, $C_{28}H_{32}N_7O_{15}P_2$ requires 768.1437.

8-(4-Methylphenyl)-NAD (3d). The triethylammonium (TEA) salt of the title compound was obtained as a glassy solid from **2** (15.4 mg, 20.7 μmol) and 4-methylphenylboronic acid in 64% yield (12.1 mg, 1.0 equiv of TEA) according to general synthetic method 1 (reaction time, 20 min). HPLC: 13.91 min (99%). δ_H (400 MHz, D_2O) 2.34 (3H, s, Me), 4.12−4.42 (8H, m, H-3′, H-3″, H-4′, H-4″, H-5′, H-5″), 4.48 (1H, t, J = 5.6 Hz, H-2″), 5.17 (1H, t, J = 5.6 Hz, H-2′), 5.79 (1H, d, J = 6.0 Hz, H-1′), 5.89 (1H, d, J = 5.2 Hz, H-1″), 7.25 (2H, d, J = 8.4 Hz, Ph), 7.39 (2H, d, J = 8.0 Hz, Ph), 8.16 (1H, t, J = 7.0 Hz, Hn_5), 8.16 (1H, s, H-2), 8.72 (1H, d, J = 8.4 Hz, Hn_4), 9.08 (1H, d, J = 6.0 Hz, Hn_6), 9.22 (1H, s, Hn_2); δ_C (125 MHz, D_2O) 21.1, 65.4, 66.4, 70.0, 71.0, 71.1, 78.2, 83.5, 87.6, 89.7, 100.7, 118.9, 125.1, 129.6, 129.7, 130.3, 134.3, 140.6, 142.7, 143.1, 146.5, 150.7, 152.8, 153.5, 155.4, 166.2; δ_P (121 MHz, D_2O) −11.8 (d, $J_{P,P}$ = 20.6 Hz), −11.3 (d, $J_{P,P}$ = 19.4 Hz). m/z (ESI) 752.1501, $[M - H]^-$, $C_{28}H_{32}N_7O_{14}P_2$ requires 752.1488.

8-(Furan-2-yl)-NAD (3e). The triethylammonium (TEA) salt of the title compound was obtained as a glassy solid from **2** (15.8 mg, 21.3 μmol) and furan-2-ylboronic acid in 45% yield (8.0 mg, 0.9 equiv of TEA) according to general synthetic method 1 (reaction time, 20 min). HPLC: 8.46 min (98%). δ_H (400 MHz, D_2O) 4.06−4.40 (8H, m, H-3′, H-3″, H-4′, H-4″, H-5′, H-5″), 4.61 (1H, t, J = 5.6 Hz, H-2″), 5.36 (1H, t, J = 5.6 Hz, H-2′), 5.79 (1H, d, J = 5.2 Hz, H-1′), 6.12 (1H, d, J = 6.0 Hz, H-1″), 6.65 (1H, dd, J = 1.6 and 3.2 Hz, fur3), 7.07 (1H, d, J = 3.6 Hz, fur2), 7.73 (1H, d, J = 1.2 Hz, fur4), 8.12 (1H, t, J = 7.2 Hz, Hn_5), 8.19 (1H, s, H-2), 8.66 (1H, d, J = 8.4 Hz, Hn_4), 8.96 (1H, d, J = 6.0 Hz, Hn_6), 9.17 (1H, s, Hn_2); δ_C (125 MHz, D_2O) 65.6, 66.6, 70.0, 71.2, 71.4, 78.4, 83.8, 87.8, 89.8, 100.7, 113.2, 115.9, 119.2, 129.6, 134.2, 140.4, 142.5, 143.0, 143.5, 146.2, 146.7, 150.3, 153.1, 155.3, 165.7; δ_P (121 MHz, D_2O) −11.9 (d, $J_{P,P}$ = 21.8 Hz), −11.4 (d, $J_{P,P}$ = 20.6 Hz). m/z (ESI) 728.1125, $[M - 2H]^-$, $C_{25}H_{28}N_7O_{15}P_2$ requires 728.1124.

8-(5-Formylthien-2-yl)-NAD (3f). The triethylammonium (TEA) salt of the title compound was obtained as a glassy solid from **1** (20.0 mg, 30.1 μmol) and 5-formylthien-2-ylboronic acid in 36% yield (10.2 mg, 1.7 equiv of TEA) according to general synthetic method 2 (cross-coupling time, 30 min). δ_H (400 MHz, D_2O) 4.06−4.40 (8H, m, H-3′, H-3″, H-4′, H-4″, H-5′, H-5″), 4.61 (1H, t, J = 5.6 Hz, H-2″), 5.50 (1H, t, J = 6.0 Hz, H-2′), 5.85 (1H, m, H-1′), 5.97 (1H, d, J = 5.9 Hz, H-1″), 7.64 (1H, d, J = 4.1 Hz, Th), 7.95 (1H, d, J = 4.1 Hz, Th), 8.16 (1H, dd, J = 6.4 and 7.8 Hz, Hn_5), 8.22 (1H, s, H-2), 8.69 (1H, d, J = 8.1 Hz, Hn_4), 9.02 (1H, d, J = 6.4 Hz, Hn_6), 9.21 (1H, s, Hn_2), 9.83 (1H, s, CHO); δ_P (121 MHz, D_2O) −11.9 (d, $J_{P,P}$ = 21.8 Hz), −11.4 (d, $J_{P,P}$ = 20.6 Hz). m/z (ESI) 772.0838 $[M - H]^-$, $C_{26}H_{28}N_7O_{15}P_2S$ requires 772.0845.

8-(5-Formylfuran-2-yl)-NAD (3g). The triethylammonium (TEA) salt of the title compound was obtained as a glassy solid from **1** (20.0 mg, 30.1 μmol) and 5-formylfuran-2-ylboronic acid in 41% yield (12.2 mg, 2.1 equiv of TEA) according to general synthetic method 2 (cross-coupling time, 20 min). δ_H (400 MHz, D_2O) 4.06−4.40 (8H, m, H-3′, H-3″, H-4′, H-4″, H-5′, H-5″), 4.70 (1H, t, J = 5.6 Hz, H-2″), 5.34 (1H, t, J = 5.9 Hz, H-2′), 5.83 (1H, d, J = 4.3 Hz, H-1′), 6.17 (1H, d, J = 5.3 Hz, H-1″), 7.26 (1H, d, J = 3.6 Hz, fur), 7.57 (1H, d, J = 3.8 Hz, fur), 8.12 (1H, t, J = 7.2 Hz, Hn_5), 8.19 (1H, s, H-2), 8.67 (1H, d, J = 8.4 Hz, Hn_4), 8.99 (1H, d, J = 6.0 Hz, Hn_6), 9.17 (1H, s, Hn_2), 9.57 (1H, s, CHO); δ_P (121 MHz, D_2O) −11.9 (d, $J_{P,P}$ = 21.8 Hz), −11.4 (d, $J_{P,P}$ = 20.6 Hz). m/z (ESI) 756.1059 $[M - H]^-$, $C_{26}H_{28}N_7O_{16}P_2$ requires 756.0173.

Sirtuin Bioassay. *Recombinant Expression of SIRT1 and SIRT2.* Human SIRT1 was expressed as a GST-tagged fusion protein, and human SIRT2 was expressed as a N-terminally tagged His_6 fusion protein. Both enzymes were purified as described with minor modifications.[37] Identity and purity of the produced enzymes were verified using SDS electrophoresis. The deacetylase activity of the sirtuins was dependent on the cofactor NAD and could be inhibited with the endogeneous sirtuin inhibitor nicotinamide.

Fluorescent Deacetylase Assay. All NAD derivatives were evaluated for their ability to inhibit recombinant SIRT1 and SIRT2 using a homogeneous fluorescent deacetylase assay.[38] DMSO stock solutions of the inhibitors were prepared, and defined amounts were added to an incubation mixture. The assay was carried out in 96-well plates with a reaction volume per well of 60 μL, containing enzyme (SIRT1 or SIRT2), the fluorescent histone deacetylase substrate ZMAL (Z-(Ac)Lys-AMC, 10.5 μM), cofactor NAD (500 μM), and inhibitor. Initially, inhibitors

were tested at 50 μM only. Inhibitors that showed inhibition greater than 50% at 50 μM were tested at a wider range of concentrations (0.1−500 μM) to allow the determination of IC_{50}. The amount of enzyme solution used was dependent on the activity of the individual enzyme preparation, which varied slightly from batch to batch. To maintain *initial state* conditions, enzyme solutions were used in amounts that gave 10−30% conversion of the substrate in the absence of inhibitor. After 4 h of incubation at 37 °C, the reaction was stopped with a solution of trypsin buffer (60 μL) containing trypsin (1 mg·mL^{-1}) from bovine pancreas (1000 (BAEE units)·mg^{-1}) and the sirtuin inhibitor nicotinamide (8 mM), and the microplate was incubated with this solution for 20 min at 37 °C. Finally, fluorescence intensity was measured in a plate reader (BMG Polarstar) with a coumarin filter (λ_{ex} = 390 nm, λ_{em}= 460 nm). The amount of remaining substrate in the positive control with inhibitor versus negative control without inhibitor was employed to calculate inhibition. All inhibition determinations were carried out at least in duplicate. IC_{50} values were calculated using GraphPad Prism software. The same assay was used to assess if the 8-substituted NAD analogues were accepted as cosubstrates by SIRT1 and SIRT2. The NAD analogues and, for direct comparison, NAD itself were tested at 500 μM, with a DMSO concentration of 8.3% (v/v). Under these conditions, only **3a** showed a detectable conversion against the blank without cofactor.

Molecular Modeling. All calculations were performed on a Pentium IV 2.2 GHz based Linux cluster (20 CPUs). The molecular structures of the NAD derivatives were generated using the crystal structure of NAD bound to archeal sirtuin Sir2-Af2 (PDB code 1yc2) and the MOE modeling package (Chemical Computing Group). The modified NAD and AMP derivatives were energy minimized using the MMFF94s force field and the conjugate gradient method until the default derivative convergence criterion of 0.01 kcal/(mol·Å) was met. The structure of the human SIRT2 protein was taken from the Protein Databank (PDB code 1j8f, monomer B). Docking of the inhibitors was carried out using the program GOLD, version 4.1,[39] and default settings (Cambridge Crystallographic Data Centre). All torsion angles in each inhibitor were allowed to rotate freely. The binding site was defined on Phe620 with a radius of 15 Å. Goldscore was chosen as fitness function. For each molecule 30 docking runs were performed. The resulting solutions were clustered on the basis of the heavy atom rmsd values (1 Å). The top-ranked poses for each ligand were retained and analyzed graphically within MOE 2006.08 (Chemical Computing Group). In addition to the docking complexes derived for human SIRT2, the archaeal Sir2-Af2 crystal structure was used for the current investigation to inspect the NAD−enzyme interaction. These docking results suggest that in general the additional substituent in position 8 is tolerated at the active site of SIRT2. A comparison of the SIRT2 docking results for the NAD derivatives modified in position 8, and the X-ray structure of the archeal homologue Sir2-Af2 in complex with NAD, shows that the ligands are involved in the same hydrogen bonds to the residues of the binding pocket (Figure S4, Supporting Information).

■ ASSOCIATED CONTENT

Supporting Information. Chromatography conditions; protocols for the synthesis of **4a**, **4b**, **5a**, and **5b**; stability testing of **2**; ^{1}H, ^{13}C, and ^{31}P NMR spectra; Figures S1−S6; and docking scores. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

ADPR, adenosine diphosphate ribose; AMC, 7-amino-4-methylcoumarin; AMP, adenosine monophosphate; ATP, adenosine triphosphate; HRMS, high-resolution mass spectrometry; NAD, nicotinamide adenine dinucleotide; SAR, structure−activity relationships; SIRT, sirtuin; TPPTS, triphenylphosphine trisulfonate; TXPTS, tris(4,6-dimethyl-3-sulfonatophenyl)phosphine; ZMAL, Z-(Ac)Lys-AMC

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(40) When ligand selectivity is discussed, it is important to note that the SIRT1 and SIRT2 enzymes used in this study have been labeled with different tags (SIRT1 with GST; SIRT2 with His). In principle, the different tags could influence the selectivity of sirtuin inhibitors, although there are no previous reports in the literature for such an effect.