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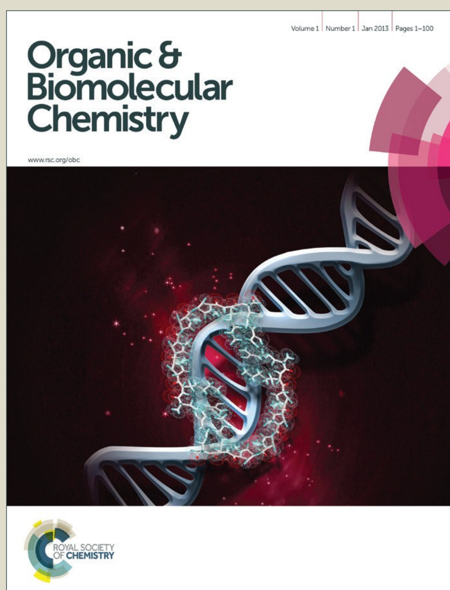

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An improved fluorogenic assay for SIRT1, SIRT2, and SIRT3

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Sirtuins are NAD-dependent lysine deacylases that play critical roles in cellular regulation and are implicated in human diseases. Modulators of sirtuins are needed as tools for investigating their biological functions and possible therapeutic applications. However, the discovery of sirtuin modulators is hampered by the lack of efficient sirtuin assays. Here we report an improved fluorogenic assay for SIRT1, SIRT2, and SIRT3 using a new substrate, a myristoyl peptide with a C-terminal aminocoumarin. The new assay has several advantages, including significantly lower substrate concentration needed, increased signal-to-background ratio, and improved Z'-factor. The novel assay thus will expedite high-throughput screening of SIRT1, SIRT2, and SIRT3 modulators.

Sirtuins, the silent information regulator 2 (Sir2) [1] family of histone deacetylases (HDACs) have seven isoforms (SIRT1−7) in mammals.[2] In contrast to the Zn^{2+}-dependent HDACs, sirtuins utilize nicotinamide adenine dinucleotide (NAD) as a co-substrate to perform deacetylation.[3] Besides lysine deacetylase activity, sirtuins have also been discovered to exhibit other enzymatic activities. For example, SIRT5 was shown to efficiently catalyze demalonylation, desuccinylation, and deglutarylation,[4-7] and SIRT6 is capable of hydrolyzing long-chain fatty acyl group on TNFα.[8] By post-translationally modifying various substrate proteins, sirtuins regulate many biological pathways and exert their effects on metabolism,[6, 9] genome stability,[10] and longevity.[11] Therefore, they are considered potential therapeutic targets for a variety of diseases, including diabetes,[12] cardiac disease,[13] and cancers.[14]

The Important biological function of sirtuins have sparked interest to develop small molecule modulators that can regulate their activity.[15, 16] Certain sirtuin inhibitors showed ability to inhibit cancer cell growth[17] and induce cancer cell-specific apoptosis.[18] Sirtuin activators could potentially be used to treat diabetes[12] and promote longevity,[11] although the effects of sirtuin activators are still under debate.[19-21] SIRT2-specific inhibitor shows promising effects in Parkinson's disease[22] and Huntington's disease.[23] Thus, developing sirtuin modulators is of great interest in both basic biological studies and therapeutic applications.

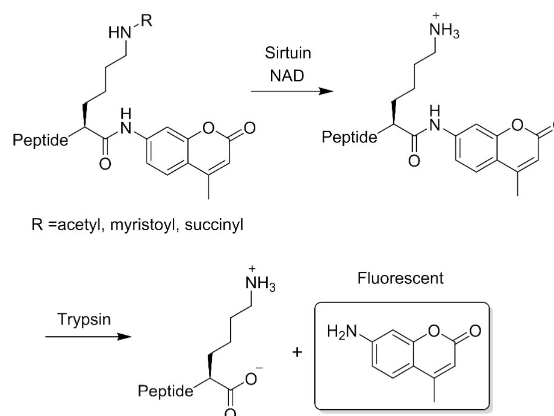

Figure 1. Principle of two-step direct fluorescence assays of sirtuins.

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1 (ZMAL, reported as SIRT1-3 substrate) **2** (R= Ac; $p53_{379-382}$, marketed as SIRT1 substrate) **3** (R= Ac; $p53_{317-320}$, marketed as SIRT2, 3 substrate) **4** (R= Myr; $p53_{317-320}$)

5 (R= Myr; TNF-α_{15-20}, reported as SIRT6 substrate) **6** (reported as SIRT5 substrate)

Figure 2. Fluorogenic substrates for direct fluorescence assays of sirtuins.

To facilitate the development of potent sirtuin modulators, many assays have been developed for the detection of sirtuin activity,[24, 25] including radioisotope-labelled assays,[26] high-performance liquid chromatography (HPLC)-based assays,[4, 8] mass spectrometry (MS)-based assays,[27, 28] and fluorescence-based assays. Fluorescence-based assays which include direct fluorescence assays,[29, 30] fluorescence polarization assays,[12] and fluorescence resonance energy transfer (FRET)-based assays.[31-34] Fluorescence-based assays are most suitable for high-throughput screening of sirtuin modulators.[11, 12] The fluorogenic substrates of direct fluorescence assays are based on a peptidic substrate of sirtuin comprising an ε-acylated lysyl moiety and a 7-amino-4-methylcoumarin moiety (AMC) at the C terminus of the peptide chain as a fluorescent tag (Figure 1).[29, 30] The acyl group can be acetyl, myristoyl, or succinyl groups depending on the target sirtuins. In the presence of the co-substrate NAD, sirtuins can remove the acyl modification from the lysine side chain, revealing the free lysine side chain. The resulting lysine peptide can then be cleaved by trypsin to release the fluorophore, allowing facile detection of sirtuin enzymatic activity by reading the fluorescence (Figure 1).

A Cbz-protected lysine with acetyl group and AMC was initially reported as a SIRT1 substrate (**1**, ZMAL, Figure 2),[35] which is then used as the substrate for SIRT2 and SIRT3 assay.[36-38] A p53-based peptide with acetyl-lysine and C-terminal AMC was developed for detecting the activity of SIRT1 (**2**, $p53_{379-382}$, Figure 2). Later, another p53-based peptide was applied to SIRT2 and SIRT3 assays (**3,** $p53_{317-320}$, Figure 2). In addition, a single lysine with succinyl group and AMC was reported as a SIRT5 substrate (**6,** Figure 2).[39] Recently, SIRT1-3 and 6 were found to efficiently remove long chain fatty acyl groups from lysine residues on proteins.[8, 40, 41] We further demonstrated that a myristoyl-lysine fluorogenic peptide (**5**, TNF-α_{15-20}, Figure 2) is a much better substrate for detecting the activity of SIRT6.[42]

One of the criteria for an ideal enzymatic assay is a low K_m value for the substrate, which renders the use of low substrate concentrations and decreases the reagent cost.[43] The K_m value of SIRT2 for a myristoyl peptide (0.24 μM) is much lower than

R = myristoyl

4

Scheme 1. Synthesis of substrates **4**. Reagents and conditions: (a) standard solid phase peptide synthesis; (b) ClCOO^{i}Bu, *N*-methylmorpholine, 7-amino-4-methyl-coumarin, DMF; (c) 1% triisopropylsilane in TFA.

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Table 1. Kinetic data for sirtuins against different fluorogenic substrates.

	SIRT1			SIRT2			SIRT3		
	K_m (μM)	k_{cat} (10^{-3}s^{-1})	k_{cat}/K_m (s^{-1}M^{-1})	K_m (μM)	k_{cat} (10^{-3}s^{-1})	k_{cat}/K_m (s^{-1}M^{-1})	K_m (μM)	k_{cat} (10^{-3}s^{-1})	k_{cat}/K_m (s^{-1}M^{-1})
3	> 333[a]	ND[a]	1.2×10^2	> 333[b]	ND[a]	18.8	> 333[b]	ND[a]	7.3
4	35.3 ± 4.5	17 ± 1.1	4.8×10^2	< 0.50[b]	10 ± 0.5	> 2.0×10^4	3.6 ± 1.2	2.7 ± 0.3	7.5×10^2
5	23.8 ± 3.9	19 ± 2.1	8.0×10^2	< 0.50[b]	12 ± 0.7	> 2.4×10^4	3.6 ± 1.3	3.5 ± 0.4	9.9×10^2

[a] The K_m and k_{cat} could not be determined due to the linear relationship at the concentration range (5.2-333 μM) used, but k_{cat}/K_m can be obtained from the slope of the linear plot (Figure S1a).
[b] The value is 51 μM for SIRT2 and 323 μM for SIRT3 provided in the commercial assay kit.
[c] The K_m could not be determined due to the detection limit of the HPLC assay. The lowest substrate concentration used was 0.5 μM.

that for an acetyl peptide (19.00 μM).[41] Based on this finding, we suspected that a myrsitoyl lysine peptides may be a better substrate and provide a better SIRT1, SIRT2, or SIRT3 assay.

To test this, a p53$_{317\text{-}320}$ peptide with myristol-lysine and C-terminal AMC (substrate **4**, Figure 2) was synthesized using standard solid phase peptide synthesis, followed by AMC coupling and deprotection (Scheme 1).[42] We first determined the kinetic constants of sirtuins against **4** and our previously reported SIRT6 substrate **5**. Using an HPLC-based assay, we measured the initial velocity, V_0, as a function of substrate concentration [S] and the data were fitted to the Michaelis–Menten equation (Figure S1) to give the K_m and k_{cat} values as shown in Table 1. The K_m of myristoyl substrates **4** and **5** for SIRT1, SIRT2, and SIRT3 are much lower than that of the commercial acetyl substrate **3**, suggesting that the myristoyl peptides have better affinities toward these sirtuins. The low K_m of myristoyl substrates is likely due to the large hydrophobic pockets of SIRT1, 2, and 3, which can accommodate the myristoyl group[41].

We then compared the fluorescence increase in the two-step sirtuin assay with different fluorogenic substrates (**3**, **4**, and **5**). We initially used the conditions specified in the commercial sirtuin assay kit. We incubated 125 μM of the fluorogenic substrates with 1 μM sirtuins (SIRT1, 2, and 3) and 3 mM NAD at 37 °C for 45 min, and then added the stop/developer solution containing trypsin and nicotinamide for 30 min at room temperature to release the fluorophore. For the commercial substrate **3**, the increase in fluorescence was less than 10-fold in SIRT1−3 reactions, which was in agreement with previous results.[39] For SIRT1 and SIRT3, substrate **4** and **5** gave slightly better fluorescence increase than substrate **3** (Figure 3). However, for SIRT2, substrate **3** gave better increase in fluorescence than substrate **4** and **5** (Figure 3), but substrate **3** also had the highest fluorescence background without addition of sirtuins (Figure S2b).

Since the K_m values of SIRT2 and SIRT3 against the myristoyl substrates **4** and **5** are less than 10 μM, we then used 10 μM of different fluorogenic substrates to compare the fluorescence increase. The commercial substrate **3** still had the highest fluorescence background (Figure S2c). Substrate **4** and **5** both gave higher fluorescence after SIRT1-3 reactions than substrates **3** (Figure 4b). After dividing by the background signal, substrate **4** gave 55-, 85-, and 43-fold increase in fluorescence in SIRT1, SIRT2, and SIRT3 reactions, respectively (Figure 4). Meanwhile, the commercial substrate **3** only gave 13-, 6-, and 5-fold increase in fluorescence in SIRT1, SIRT2, and SIRT3 reactions, respectively. Substrate **5** also gave higher

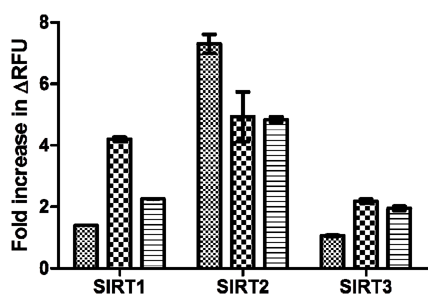

Figure 3. Fold increase in fluorescence with different substrates at 125 μM in the two-step sirtuin assay.

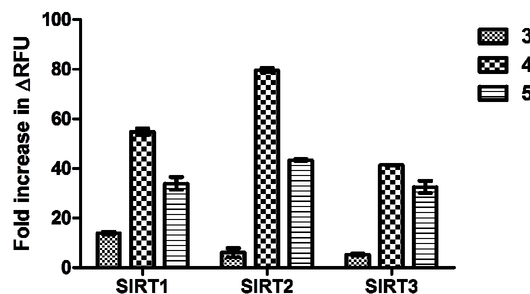

Figure 4. Fold increase in fluorescence with different substrates at 10 μM in the two-step sirtuin assay.

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Table 2. Evaluation of the fluorogenic assay by measuring signal-to-background (S/B) ratio and Z'-factor at various substrate concentrations.

		Substrate **4** concentration (μM)			
		2.5	5	10	20
SIRT1	S/B	21	75	48	30
	Z'-factor	-0.45	0.98	0.83	0.68
SIRT2	S/B	60	165	80	45
	Z'-factor	0.85	0.87	0.72	0.93
SIRT3	S/B	14	45	38	16
	Z'-factor	0.61	0.92	0.89	0.81

fluorescence increases in the assays than **3**, but compared to substrate **4** the increases were slightly smaller. The new substrate **4** not only works at lower concentration but also gives larger fold increase than the substrate **3** from the commercial assay kit.

To evaluate the quality of the sirtuin assay for future high-throughput screening use, signal to background (S/B) ratio and Z'-factor were considered.[44] S/B ratio represents assay sensitivity, and Z'-factor reflects dynamic range of the assay and the data variation. In the case of fluorogenic assay, fold increase in fluorescence is equivalent to S/B ratio. Although substrate **4** exhibited lower catalytic efficacy than **5** (Table 1), it gave higher S/B ratio and was therefore chosen for further optimization. We varied the concentration of substrate **4** and obtained the S/B ratios and Z'-factors (Table 2). In SIRT1, SIRT2, and SIRT3 assay, 5 μM of substrate **4** gave the best result. The S/B ratio were 75, 165, and 45, and the Z'-factors were 0.98, 0.87, and 0.92 in SIRT1, SIRT2, and SIRT3 assays, respectively. Compared to the use of 125 μM of substrate **3** in the commercial assay kit (S/B and Z'-factor), the significantly improved S/B ratio, the excellent Z'-factor, and the reduction of the working concentration of substrate **4** will better enable high-throughput screening.

To demonstrate that the new fluorogenic substrate **4** is suitable to detect sirtuin inhibitors, we measured the dose-dependent inhibition of sirtuins by nicotinamide. The dose-response curve was obtained by measuring the fluorescence in various concentration of nicotinamide with 5 μM of substrate **4** and 1 mM of NAD (Figure 5). The IC_{50} values of nicotinamide for SIRT1, SIRT2, and SIRT3 were determined to be 79, 138, and 84 μM, respectively. The IC_{50} values were comparable to those previously reported.[45, 46] Thus, the new fluorogenic assay provides reliable IC_{50} and should be suitable for screening unknown inhibitors of SIRT1/2/3.

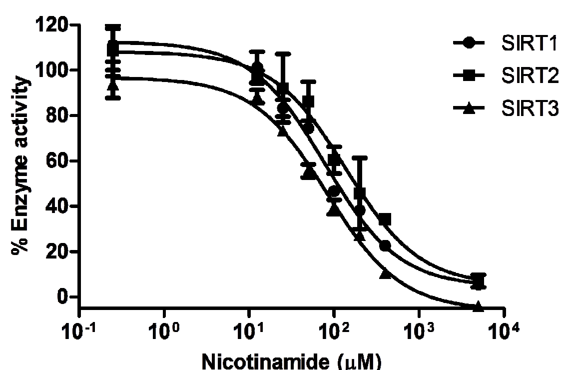

Figure 5. Dose-dependent inhibition of sirtuins by nicotinamide. IC_{50} of nicotinamide for SIRT1, SIRT2, and SIRT3 was determined to be 79, 138, and 84 μM respectively.

Conclusions

We have developed an improved fluorogenic assay for SIRT1, SIRT2, and SIRT3 using a new myristoyl peptide with C-terminal AMC as the substrate. The new substrate, **4**, gave the much excellent S/B ratios and Z'-factors compared to the currently available commercial assay. The new substrate also dramatically decreases the substrate concentration needed, and thus will decrease reagent cost in high-throughput screening. The assay thus will help to discover new sirtuin modulators via high-throughput screening and facilitate biological and pharmacological studies of sirtuins.

Notes and references

1. J. S. Smith, C. B. Brachmann, I. Celic, M. A. Kenna, S. Muhammad, V. J. Starai, J. L. Avalos, J. C. Escalante-Semerena, C. Grubmeyer, C. Wolberger and J. D. Boeke, *Proc. Natl. Acad. Sci. U.S.A.*, 2000, **97**, 6658.
2. R. A. Frye, *Biochem. Biophys. Res. Commun.*, 2000, **273**, 793.
3. S. Imai, C. M. Armstrong, M. Kaeberlein and L. Guarente, *Nature*, 2000, **403**, 795.
4. J. T. Du, Y. Y. Zhou, X. Y. Su, J. J. Yu, S. Khan, H. Jiang, J. Kim, J. Woo, J. H. Kim, B. H. Choi, B. He, W. Chen, S. Zhang, R. A. Cerione, J. Auwerx, Q. Hao and H. N. Lin, *Science*, 2011, **334**, 806.
5. M. J. Rardin, W. He, Y. Nishida, J. C. Newman, C. Carrico, S. R. Danielson, A. Guo, P. Gut, A. K. Sahu, B. Li, R. Uppala, M. Fitch, T. Riiff, L. Zhu, J. Zhou, D. Mulhern, R. D. Stevens, O. R. Ilkayeva, C. B. Newgard, M. P. Jacobson, M. Hellerstein, E. S. Goetzman, B. W. Gibson and E. Verdin, *Cell Metab.*, 2013, **18**, 920.
6. J. Park, Y. Chen, D. X. Tishkoff, C. Peng, M. J. Tan, L. Z. Dai, Z. Y. Xie, Y. Zhang, B. M. M. Zwaans, M. E. Skinner, D. B. Lombard and Y. M. Zhao, *Mol. Cell*, 2013, **50**, 919.
7. M. J. Tan, C. Peng, K. A. Anderson, P. Chhoy, Z. Y. Xie, L. Z. Dai, J. Park, Y. Chen, H. Huang, Y. Zhang, J. Ro, G. R. Wagner, M. F. Green, A. S. Madsen, J. Schmiesing, B. S. Peterson, G. F. Xu, O. R. Ilkayeva, M. J. Muehlbauer, T. Braulke, C. Muhlhausen, D. S. Backos, C. A. Olsen, P. J. McGuire, S. D. Pletcher, D. B. Lombard, M. D. Hirschey and Y. M. Zhao, *Cell Metab.*, 2014, **19**, 605.
8. H. Jiang, S. Khan, Y. Wang, G. Charron, B. He, C. Sebastian, J. T. Du, R. Kim, E. Ge, R. Mostoslavsky, H. C. Hang, Q. Hao and H. N. Lin, *Nature*, 2013, **496**, 110.

Organic & Biomolecular Chemistry Accepted Manuscript

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9. R. H. Houtkooper, E. Pirinen and J. Auwerx, *Nat. Rev. Mol. Cell Biol.*, 2012, **13**, 225.
10. H. Jing and H. Lin, *Chem. Rev.*, 2015, **115**, 2350.
11. K. T. Howitz, K. J. Bitterman, H. Y. Cohen, D. W. Lamming, S. Lavu, J. G. Wood, R. E. Zipkin, P. Chung, A. Kisielewski, L. L. Zhang, B. Scherer and D. A. Sinclair, *Nature*, 2003, **425**, 191.
12. J. C. Milne, P. D. Lambert, S. Schenk, D. P. Carney, J. J. Smith, D. J. Gagne, L. Jin, O. Boss, R. B. Perni, C. B. Vu, J. E. Bemis, R. Xie, J. S. Disch, P. Y. Ng, J. J. Nunes, A. V. Lynch, H. Y. Yang, H. Galonek, K. Israelian, W. Choy, A. Iffland, S. Lavu, O. Medvedik, D. A. Sinclair, J. M. Olefsky, M. R. Jirousek, P. J. Elliott and C. H. Westphal, *Nature*, 2007, **450**, 712-716.
13. S. Matsushima and J. Sadoshima, *Am. J. Physiol. Heart Circ. Physiol.*, 2015, **309**, H1375.
14. M. Roth and W. Y. Chen, *Oncogene*, 2014, **33**, 1609.
15. J. M. Villalba and F. J. Alcain, *BioFactors*, 2012, **38**, 349-359.
16. J. Hu, H. Jing and H. N. Lin, *Future Med. Chem.*, 2014, **6**, 945-966.
17. B. Heltweg, T. Gatbonton, A. D. Schuler, J. Posakony, H. Li, S. Goehle, R. Kollipara, R. A. Depinho, Y. Gu, J. A. Simon and A. Bedalov, *Cancer Res.*, 2006, **66**, 4368.
18. E. Lara, A. Mai, V. Calvanese, L. Altucci, P. Lopez-Nieva, M. L. Martinez-Chantar, M. Varela-Rey, D. Rotili, A. Nebbioso, S. Ropero, G. Montoya, J. Oyarzabal, S. Velasco, M. Serrano, M. Witt, A. Villar-Garea, A. Imhof, J. M. Mato, M. Esteller and M. F. Fraga, *Oncogene*, 2009, **28**, 781.
19. M. T. Borra, B. C. Smith and J. M. Denu, *J. Biol. Chem.*, 2005, **280**, 17187.
20. M. Kaeberlein, T. McDonagh, B. Heltweg, J. Hixon, E. A. Westman, S. D. Caldwell, A. Napper, R. Curtis, P. S. DiStefano, S. Fields, A. Bedalov and B. K. Kennedy, *J. Biol. Chem.*, 2005, **280**, 17038.
21. M. Pacholec, J. E. Bleasdale, B. Chrunyk, D. Cunningham, D. Flynn, R. S. Garofalo, D. Griffith, M. Griffor, P. Loulakis, B. Pabst, X. Y. Qiu, B. Stockman, V. Thanabal, A. Varghese, J. Ward, J. Withka and K. Ahn, *J. Biol. Chem.*, 2010, **285**, 8340.
22. T. F. Outeiro, E. Kontopoulos, S. M. Altmann, I. Kufareva, K. E. Strathearn, A. M. Amore, C. B. Volk, M. M. Maxwell, J. C. Rochet, P. J. McLean, A. B. Young, R. Abagyan, M. B. Feany, B. T. Hyman and A. G. Kazantsev, *Science*, 2007, **317**, 516-519.
23. R. Luthi-Carter, D. M. Taylor, J. Pallos, E. Lambert, A. Amore, A. Parker, H. Moffitt, D. L. Smith, H. Runne, O. Gokce, A. Kuhn, Z. Xiang, M. M. Maxwell, S. A. Reeves, G. P. Bates, C. Neri, L. M. Thompson, J. L. Marsh and A. G. Kazantsev, *Proc. Natl. Acad. Sci. U.S.A.*, 2010, **107**, 7927.
24. Y. Li, T. Liu, S. Liao, Y. Li, Y. Lan, A. Wang, Y. Wang and B. He, *Biochem. Biophys. Res. Commun.*, 2015, **467**, 459.
25. M. Schutkowski, F. Fischer, C. Roessler and C. Steegborn, *Expert Opin. Drug Discov.*, 2014, **9**, 183.
26. M. T. Borra and J. M. Denu, *Meth. Enzymol.*, 2004, **376**, 171.
27. P. T. Rye, L. E. Frick, C. C. Ozbal and W. A. Lamarr, *J. Biomol. Screen.*, 2011, **16**, 1217.
28. K. Patel, J. Sherrill, M. Mrksich and M. D. Scholle, *J. Biomol. Screen.*, 2015, **20**, 842.
29. D. Wegener, C. Hildmann, D. Riester and A. Schwienhorst, *Anal. Biochem.*, 2003, **321**, 202.
30. D. Wegener, F. Wirsching, D. Riester and A. Schwienhorst, *Chem. Biol.*, 2003, **10**, 61.
31. P. A. Marcotte, P. R. Richardson, J. Guo, L. W. Barrett, N. Xu, A. Gunasekera and K. B. Glaser, *Anal. Biochem.*, 2004, **332**, 90.
32. D. Riester, C. Hildmann, A. Schwienhorst and F. J. Meyer-Almes, *Anal. Biochem.*, 2007, **362**, 136.
33. Y. Li, W. Huang, L. You, T. Xie and B. He, *Bioorg. Med. Chem. Lett.*, 2015, **25**, 1671.
34. Y. Li, L. You, W. Huang, J. Liu, H. Zhu and B. He, *Eur. J. Med. Chem.*, 2015, **96**, 245.
35. B. Heitweg, F. Dequiedt, E. Verdin and M. Jung, *Anal. Biochem.*, 2003, **319**, 42.
36. P. Di Fruscia, K. K. Ho, S. Laohasinnarong, M. Khongkow, S. H. B. Kroll, S. A. Islam, M. J. E. Sternberg, K. Schmidtkunz, M. Jung, E. W. F. Lam and M. J. Fuchter, *Medchemcomm*, 2012, **3**, 373.
37. T. Rumpf, M. Schiedel, B. Karaman, C. Roessler, B. J. North, A. Lehotzky, J. Olah, K. I. Ladwein, K. Schmidtkunz, M. Gajer, M. Pannek, C. Steegborn, D. A. Sinclair, S. Gerhardt, J. Ovadi, M. Schutkowski, W. Sippl, O. Einsle and M. Jung, *Nat. Commun.*, 2015, **6**, 6263.
38. B. Maurer, T. Rumpf, M. Scharfe, D. A. Stolfa, M. L. Schmitt, W. J. He, E. Verdin, W. Sippl and M. Jung, *ACS Med. Chem. Lett.*, 2012, **3**, 1050.
39. A. S. Madsen and C. A. Olsen, *J. Med. Chem.*, 2012, **55**, 5582.
40. J. L. Feldman, J. Baeza and J. M. Denu, *J. Biol. Chem.*, 2013, **288**, 31350.
41. Y. B. Teng, H. Jing, P. Aramsangtienchai, B. He, S. Khan, J. Hu, H. Lin and Q. Hao, *Sci. Rep.*, 2015, **5**, 8529.
42. J. Hu, B. He, S. Bhargava and H. Lin, *Org. Biomol. Chem.*, 2013, **11**, 5213.
43. J. W. London, L. M. Shaw, D. Fetterolf and D. Garfinkel, *Clin. Chem.*, 1975, **21**, 1939.
44. J. H. Zhang, T. D. Y. Chung and K. R. Oldenburg, *J. Biomol. Screen.*, 1999, **4**, 67.
45. F. Fischer, M. Gertz, B. Suenkel, M. Lakshminarasimhan, M. Schutkowski and C. Steegborn, *Plos One*, 2012, **7**, e45098.
46. X. Guan, P. Lin, E. Knoll and R. Chakrabarti, *Plos One*, 2014, **9**, e107729.

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