

# Design of a Hyperpolarized Molecular Probe for Detection of Aminoamidase N Activity

Ryunosuke Hata, Hiroshi Nonaka, Yoichi Takakusagi, Kazuhiro Ichikawa, and Shinsuke Sando\*

**Abstract:** Aminoamidase N (APN) is an important enzyme that is involved in tumor angiogenesis. Detection of APN activity can thus lead to early diagnosis and elucidation of tumor development. Although some molecular probes for APN have been developed, the detection of APN activity in opaque biological samples remains a challenge. To this end, we designed a hyperpolarized NMR probe [1-<sup>13</sup>C]Ala-NH<sub>2</sub> which satisfies the prerequisites for APN detection, namely, sufficient retention of the hyperpolarized state, a high reactivity to APN, and an APN-induced chemical shift change. The [1-<sup>13</sup>C]Ala-NH<sub>2</sub> probe allowed sensitive detection of APN activity using <sup>13</sup>C NMR spectroscopy.

Aminoamidase N (APN) is an enzyme that catalyzes the hydrolysis of peptides or proteins and releases N-terminal hydrophobic amino acids such as alanine.<sup>[1]</sup> APN plays a number of essential roles in terms of physiological function. APN is also expected to be a tumor biomarker because the function of APN is associated with tumor invasion, metastasis, and angiogenesis.<sup>[2]</sup> Therefore, the development of a molecular probe that can be used to detect APN activity has been in high demand.

APN-binding probes that are ligands labeled with a fluorophore or radioisotope have been used to image APN expressed within the cell and in vivo.<sup>[3–5]</sup> However, such probes require washing or excretion of unbound probes, which prevents their use in real-time analysis. Furthermore, the sensing function of these probes does not directly reflect the enzymatic activity of APN. The enzymatic activity has potential to be a reliable parameter in pathological analy-

sis.<sup>[6,7]</sup> To this end, a molecular probe that can be used to analyze the enzymatic activity of APN is required.

For this purpose, enzymatic-reaction-based probes, which produce optical readouts upon reaction with APN, have been developed.<sup>[8,9]</sup> However, the detection of APN activity in biological samples, such as tissues, their homogenates, and living animals, remains challenging because visible light is absorbed or scattered by biological material.

NMR molecular probes have good potential for use in the detection of enzymatic activity in such opaque biological samples.<sup>[10]</sup> Hyperpolarized NMR probes, the NMR sensitivities of which are dramatically enhanced by a nuclear polarization process, have emerged as a particularly promising tool.<sup>[11]</sup> A representative probe is hyperpolarized [1-<sup>13</sup>C]pyruvic acid, which allows even in vivo sensing of lactate dehydrogenase activity by <sup>13</sup>C chemical shift analysis.<sup>[12]</sup> Despite its potential use, the number of hyperpolarized NMR probes that have been developed so far remains limited.<sup>[13–15]</sup> This is because of the stringent chemical and physical requirement for hyperpolarized NMR probes (see below). Herein, we report the design and synthesis of the first hyperpolarized NMR probe that can be used to analyze APN activity.

APN catalyzes the hydrolysis of peptides at an N-terminal hydrophobic amino acid residue, mainly alanine, methionine, and leucine. When designing the hyperpolarized NMR probe for use in assessing APN activity, a straightforward approach is to use a peptide structure such as dipeptide [1-<sup>13</sup>C]Ala-Xaa (Figure 1, top). This dipeptide appeared to be ideal because it reacts with APN and would work as a <sup>13</sup>C NMR chemical-shift-switching probe. However, the use of such dipeptide-type probes presents at least two potential problems, the first of which being selectivity. Given the range of peptidases that are present in the body, the probe peptides could be a substrate for other peptidases in addition to APN. In the case of dipeptides, carboxypeptidases (CPs) and dipeptidases (DPs) are also assumed to react, resulting in low APN selectivity and non-negligible levels of false positive signals. The second potential problem presented by the use of dipeptide-type probes is the hyperpolarization lifetime. Peptide structures tend to have a short hyperpolarization lifetime.<sup>[16]</sup> The lifetime is directly related to spin-lattice relaxation time (*T*<sub>1</sub>). Therefore, the designed NMR probe should have a long *T*<sub>1</sub> value that is sufficient for a delivery to the target site, an enzymatic reaction, and NMR measurements.

To address these problems, we designed [1-<sup>13</sup>C]Ala-NH<sub>2</sub> (Figure 1, bottom) as a probe. To avoid reaction with endogenous peptidases except APN, we focused on the

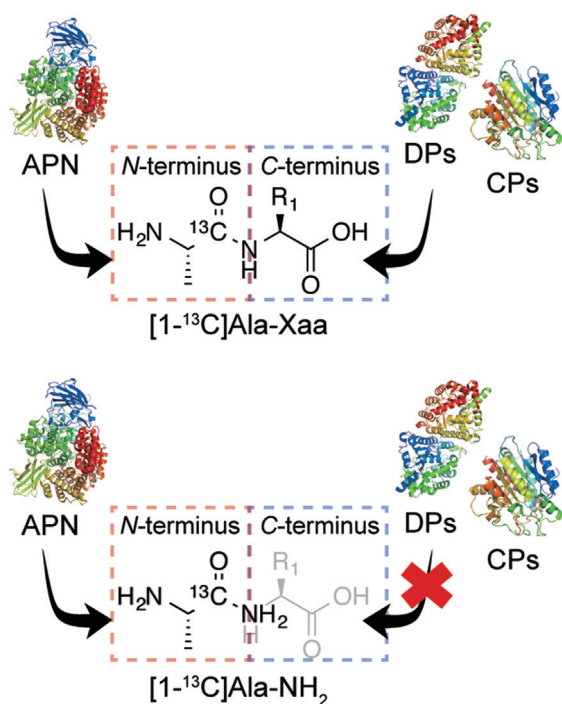
[\*] Dr. H. Nonaka, Prof. Dr. S. Sando  
Department of Chemistry and Biotechnology  
Graduate School of Engineering, The University of Tokyo  
7-3-1 Hongo, Bunkyo-ku, Tokyo 113–8656 (Japan)  
E-mail: ssando@chembio.t.u-tokyo.ac.jp

R. Hata  
Department of Chemistry and Biochemistry  
Graduate School of Engineering, Kyushu University  
744 Moto-oka, Nishi-ku, Fukuoka 819-0395 (Japan)

Dr. Y. Takakusagi, Prof. Dr. K. Ichikawa  
Incubation Center for Advanced Medical Science  
Kyushu University  
3-1-1 Maidashi, Higashi-ku, Fukuoka 812–8582 (Japan)

Prof. Dr. K. Ichikawa  
Innovation Center for Medical Redox Navigation  
Kyushu University  
3-1-1 Maidashi, Higashi-ku, Fukuoka 812–8582 (Japan)

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201509457>.

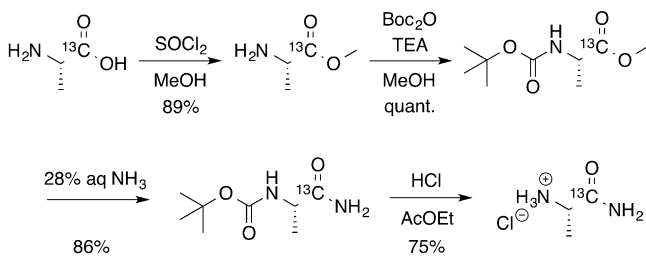


**Figure 1.** Design of APN-selective probe  $[1-^{13}\text{C}]\text{Ala-NH}_2$ . Model structures of APN, DPs, and CPs were from PDB 3B34, 1ITU, and 2PCU.

carboxylic acid of the dipeptide structure. CPs recognize the C-terminal carboxylic acid in substrates, as its name implies.<sup>[17]</sup> Some reports suggest that DPs also require the C-terminal carboxylic acid moiety to perform hydrolysis.<sup>[18]</sup> Based on these reports, we focused on  $[1-^{13}\text{C}]\text{Ala-NH}_2$  having a primary amide group because this potential probe has no C-terminal carboxylic acid, and we anticipated that it would show a high selectivity to APN over CPs and DPs. Additionally, a long hyperpolarization lifetime for this compound was expected because of its small size compared with dipeptides.

$[1-^{13}\text{C}]\text{Ala-NH}_2$  was synthesized from  $[1-^{13}\text{C}]\text{Ala}$  in four steps (Scheme 1). The carboxylic acid of  $[1-^{13}\text{C}]\text{Ala}$  was converted into the methyl ester, followed by protection of the amino group with the *tert*-butoxycarbonyl (Boc) group. The methyl ester was then converted into a primary amide by treatment with ammonium hydroxide. The Boc group was deprotected with hydrochloric acid to give  $[1-^{13}\text{C}]\text{Ala-NH}_2$ .

First, we determined the  $T_1$  value and APN reactivity of Ala-NH<sub>2</sub>, L-Alanyl-glycine (Ala-Gly) and L-alanyl-L-alanine (Ala-Ala), the smallest and second smallest dipeptides having



**Scheme 1.** Synthesis of  $[1-^{13}\text{C}]\text{Ala-NH}_2$ .

alanine as the N-terminal amino acid, were used as comparable probes.

As expected, Ala-NH<sub>2</sub> displayed a longer  $^{13}\text{C}$   $T_1$  value than both dipeptides. The  $T_1$  values of  $^{13}\text{C}$ -labeled substrates,  $[1-^{13}\text{C}]\text{Ala-NH}_2$ ,  $[1-^{13}\text{C}]\text{Ala-Gly}$ , and  $[1-^{13}\text{C}]\text{Ala-Ala}$ , were determined by using the inversion recovery method to be  $24.8 \pm 0.9$ ,  $16.6 \pm 0.2$ , and  $13.6 \pm 0.4$  s (9.4 T, 37°C), respectively (Table 1). The  $T_1$  value of  $[1-^{13}\text{C}]\text{Ala-NH}_2$  was 1.5–1.8 times larger than that of the dipeptides and was within the  $T_1$  range of probes that have been successfully applied for *in vivo* analysis.<sup>[13–15]</sup>

**Table 1:**  $T_1$  values and kinetic parameters for Ala-NH<sub>2</sub>, Ala-Gly, and Ala-Ala.

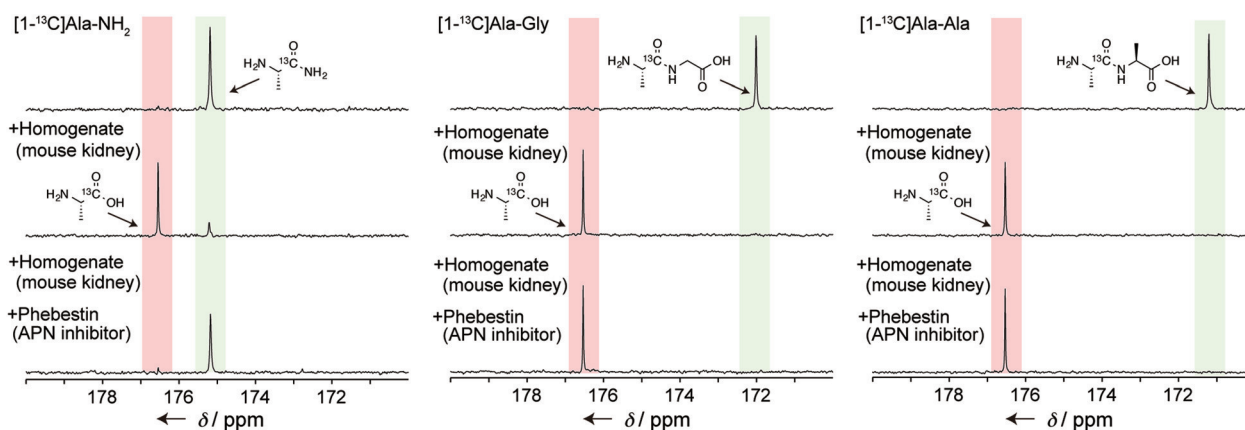
Probe	$K_m$ [mM] <sup>[a]</sup>	$k_{\text{cat}}$ [s <sup>−1</sup> ] <sup>[a]</sup>	$T_1$ [s] <sup>[b]</sup>
Ala-NH <sub>2</sub>	$9.7 \pm 1.7$	$210 \pm 10$	$24.8 \pm 0.9$
Ala-Gly	$3.6 \pm 0.4$	$74 \pm 8$	$16.6 \pm 0.2$
Ala-Ala	$2.9 \pm 0.6$	$150 \pm 10$	$13.6 \pm 0.4$

[a] Nonlabeled substrates were used to evaluate kinetic parameters. Conditions: 100 mM phosphate buffer pH 7.4 (37°C). [b] Isotopically labeled  $[1-^{13}\text{C}]\text{Ala-NH}_2$ ,  $[1-^{13}\text{C}]\text{Ala-Gly}$ , and  $[1-^{13}\text{C}]\text{Ala-Ala}$  were used. Conditions: 10 mM  $^{13}\text{C}$ -labeled compounds in 90 mM sodium phosphate buffer (pH 7.4) containing 10% D<sub>2</sub>O (9.4 T, 37°C).

Kinetic parameters for APN,  $k_{\text{cat}}$  and  $K_m$ , were determined by using the non-isotopically labeled substrates Ala-NH<sub>2</sub>, Ala-Gly, and Ala-Ala (Table 1). The  $K_m$  values revealed that the affinity of Ala-NH<sub>2</sub> for rat APN is slightly less than the affinity of the dipeptides. On the other hand, interestingly, the  $k_{\text{cat}}$  value of Ala-NH<sub>2</sub> was larger than those of the dipeptides. In hyperpolarized NMR experiments, a large  $k_{\text{cat}}$  value is required to afford detectable amounts of reaction products within a limited hyperpolarization lifetime. The detected large  $k_{\text{cat}}$  value ( $210 \text{ s}^{-1}$ ) would satisfy this requirement.

Next, the enzyme selectivity was evaluated by using  $^{13}\text{C}$  NMR spectroscopic analysis (Figure 2).  $[1-^{13}\text{C}]\text{Ala-NH}_2$ ,  $[1-^{13}\text{C}]\text{Ala-Gly}$ , and  $[1-^{13}\text{C}]\text{Ala-Ala}$  afforded  $^{13}\text{C}$  NMR signals at  $\delta = 175.2$ , 172.0, and 171.2 ppm, respectively (Figure 2, top line). These substrates were each mixed with a homogenate of mouse kidney, which is known to contain APN and other peptidases.<sup>[19–21]</sup> After incubation, the  $^{13}\text{C}$  NMR spectra of all three samples showed the same new signal at  $\delta = 176.5$  ppm (Figure 2, middle line). This new signal was assigned as  $[1-^{13}\text{C}]\text{Ala}$ , suggesting that peptidases in the kidney homogenate hydrolyzed all of these substrates to release  $[1-^{13}\text{C}]\text{Ala}$ . However, when the same reactions were conducted in the presence of phebestin (10  $\mu\text{M}$ ), which is a selective inhibitor of APN ( $\text{IC}_{50} = 0.41 \mu\text{M}$ ),<sup>[22]</sup> the reaction of  $[1-^{13}\text{C}]\text{Ala-NH}_2$  was inhibited almost completely (Figure 2, bottom), whereas  $[1-^{13}\text{C}]\text{Ala-Ala}$  and  $[1-^{13}\text{C}]\text{Ala-Gly}$  still reacted to give  $[1-^{13}\text{C}]\text{Ala}$ . These combined results indicate that  $[1-^{13}\text{C}]\text{Ala-NH}_2$  reacts selectively with APN, whereas dipeptide-based probes react with a broad range of peptidases or enzymes.

The APN selectivity of Ala-NH<sub>2</sub> was further confirmed by comparison with the known APN-selective chromogenic probe alanine-*p*-nitroanilide (Ala-pNA). The relative reac-

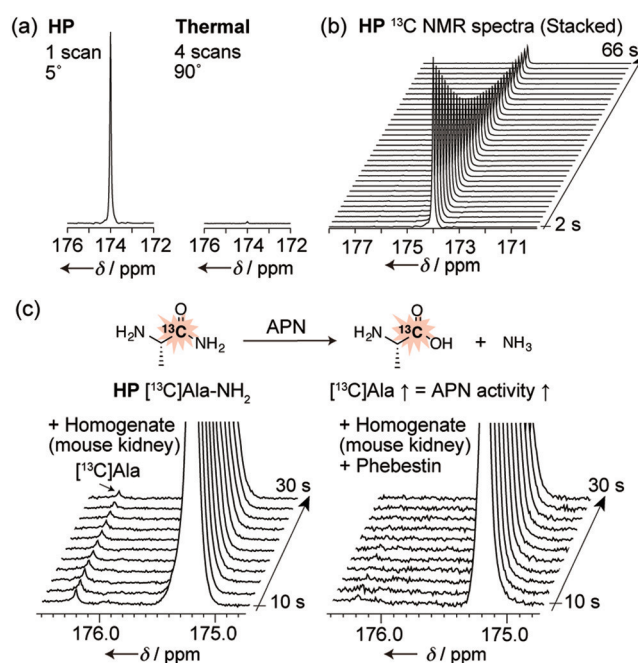


**Figure 2.**  $^{13}\text{C}$  NMR spectra of  $[1-^{13}\text{C}]\text{Ala-NH}_2$ ,  $[1-^{13}\text{C}]\text{Ala-Gly}$ , and  $[1-^{13}\text{C}]\text{Ala-Ala}$  before incubation (top line) or after incubation for 6 hours with kidney homogenate in the absence (middle line) or the presence (bottom line) of phebestin. Incubation conditions: 10 mM  $^{13}\text{C}$ -labeled substrate in 100 mM phosphate buffer (pH 7.4, 500  $\mu\text{L}$ ) containing mouse kidney homogenate (10  $\mu\text{L}$ , 39  $\text{mg mL}^{-1}$  protein by BCA assay) and 0 or 10  $\mu\text{M}$  phebestin. All solutions were mixed with 60  $\mu\text{L}$  of dioxane/ $\text{D}_2\text{O}$  = 15:1 v/v as an internal reference ( $\delta = 67.2$  ppm) before  $^{13}\text{C}$  NMR measurements (128 scans).

tivity of the Ala- $\text{NH}_2$  probe in each of the tissue homogenates (kidney, liver, pancreas, lung, spleen, and heart) was highly correlated with those of Ala-pNA. On the other hand, those of Ala-Ala and Ala-Gly showed different profiles (see Figure S1 in the Supporting Information). These results also support the conclusion that Ala- $\text{NH}_2$  is likely to be a selective substrate for APN.

With a potent APN-selective probe in hand, we then performed a proof-of-concept hyperpolarization study. The  $[1-^{13}\text{C}]\text{Ala-NH}_2$  solution containing 15 mM of the trityl radical OX63 (in  $\text{D}_2\text{O}/[\text{D}_8]\text{glycerol} = 85:15$  v/v) was hyperpolarized at 1.4 K, irradiating at 94 GHz, using HyperSense (Oxford Instruments). This sample was dissolved rapidly and subjected to  $^{13}\text{C}$  NMR spectroscopic analysis (9.4 T). An enhanced NMR signal was clearly detected after a single scan (Figure 3a, left). Compared with the thermally equilibrated  $^{13}\text{C}$  NMR spectrum (Figure 3a, right), the signal was enhanced 6200-fold. The time course of the hyperpolarized  $^{13}\text{C}$  NMR spectra is shown in Figure 3b. As a result of the relatively long  $T_1$  value, the hyperpolarized signal could be detected for over 60 seconds by repeated single NMR scans. Curve fitting to the signal decay gave  $T_1 = 30.1$  s, which was consistent with the  $T_1$  value obtained for the thermally equilibrated sample ( $T_1 = 30.2 \pm 0.6$  s, inversion recovery, 10 mM  $[1-^{13}\text{C}]\text{Ala-NH}_2$ , 250  $\text{mg L}^{-1}$  EDTA aqueous solution containing 10%  $\text{D}_2\text{O}$ , 9.4 T, 37  $^\circ\text{C}$ ; EDTA = ethylenediaminetetraacetic acid).

Finally, we applied the hyperpolarized probe for the detection of APN activity in kidney homogenate (Figure 3c). When the hyperpolarized  $[1-^{13}\text{C}]\text{Ala-NH}_2$  probe was added to mouse kidney homogenate, the probe produced a new NMR signal corresponding to  $[1-^{13}\text{C}]\text{Ala}$  as a reporting product. In contrast, in the presence of phebestin, the signal of  $[1-^{13}\text{C}]\text{Ala}$  was largely absent. The signal of hyperpolarized  $[1-^{13}\text{C}]\text{Ala}$  seemed to reach a plateau (Figure 3c). This is probably because  $[1-^{13}\text{C}]\text{Ala}$  was gradually produced by the enzymatic reaction during the measurement time. It is highly likely that the sum of the hyperpolarized signal decay and enzymatic



**Figure 3.** a) Hyperpolarized (HP, left) and thermally equilibrated (thermal, right)  $^{13}\text{C}$  NMR signals of  $[1-^{13}\text{C}]\text{Ala-NH}_2$  (7.5 mM in 250  $\text{mg L}^{-1}$  EDTA solution). b) Stacked hyperpolarized  $^{13}\text{C}$  NMR spectra of  $[1-^{13}\text{C}]\text{Ala-NH}_2$  (7.5 mM in 250  $\text{mg L}^{-1}$  EDTA solution), showing spectra recorded from 2–66 s (spectra recorded every 2 s, pulse angle =  $5^\circ$ ). c) Detection of APN activity using hyperpolarized  $[1-^{13}\text{C}]\text{Ala-NH}_2$ , with  $^{13}\text{C}$  NMR spectra recorded every 2 s (pulse angle =  $25^\circ$ ). The solutions of hyperpolarized  $[1-^{13}\text{C}]\text{Ala-NH}_2$  (16 mM, 3 mL) in 100 mM phosphate buffer (pH 7.4) were mixed with mouse kidney homogenate (1 mL) in the absence (left) or in the presence (right) of phebestin (5  $\mu\text{M}$ ).

production of  $[1-^{13}\text{C}]\text{Ala}$  resulted in the signal reaching a plateau. Thermally equilibrated  $^{13}\text{C}$  NMR spectra measured after these hyperpolarization experiments also support that the new resonance signal is actually the signal derived from  $[1-^{13}\text{C}]\text{Ala}$  produced (Figure S2).

At the moment, the presence of unknown enzymes that hydrolyze  $[1-^{13}\text{C}]\text{Ala-NH}_2$  cannot be excluded; however, considering its high selectivity for APN, it is reasonable to conclude that hyperpolarized  $[1-^{13}\text{C}]\text{Ala-NH}_2$  works as a selective probe for detecting APN activity.

In conclusion, we have developed  $[1-^{13}\text{C}]\text{Ala-NH}_2$  as the first hyperpolarized NMR probe for detecting APN activity. The compound satisfies prerequisites for hyperpolarized NMR analysis: sufficient retention of the hyperpolarized state, a high reactivity to APN, an APN-induced chemical shift change, and low toxicity (Figure S3). In addition, APN is a membrane protein bearing a catalytic domain outside of the cell.<sup>[1]</sup> Therefore,  $[1-^{13}\text{C}]\text{Ala-NH}_2$  can react with APN without the need for cellular uptake. This would be also an advantage of this probe in practical applications. Proof-of-concept experiments demonstrated the detection of APN activity in mouse kidney homogenate by using hyperpolarized  $[1-^{13}\text{C}]\text{Ala-NH}_2$ . The  $[1-^{13}\text{C}]\text{Xaa-NH}_2$  probe concept demonstrated here is not limited to APN, but can be applied in the design of hyperpolarized NMR probes for other aminopeptidases. We expect that probes of the type  $[1-^{13}\text{C}]\text{Xaa-NH}_2$  will open up new opportunities for the application of hyperpolarized NMR systems. Further work along these lines is under way in our laboratory.

## Acknowledgements

This work was supported by CREST, Japan Science and Technology Agency (JST). The use of HyperSense was in part supported by the funding program "Creation of Innovation Centers for Advanced Interdisciplinary Research Areas" from the JST. R.H. was funded by a fellowship from the Japan Society for the Promotion of Science. We thank Ms. Kaori Inoue for measurements of DNP-NMR.

**Keywords:** aminopeptidases · biosensors · dynamic nuclear polarization · hyperpolarization · NMR spectroscopy

**How to cite:** *Angew. Chem. Int. Ed.* **2016**, *55*, 1765–1768  
*Angew. Chem.* **2016**, *128*, 1797–1800

[1] Y. Luan, W. Xu, *Curr. Med. Chem.* **2007**, *14*, 639–647.

[2] M. Wickström, R. Larsson, P. Nygren, J. Gullbo, *Cancer Sci.* **2011**, *102*, 501–508.

- [3] K. Chen, W. Ma, G. Li, J. Wang, W. Yang, L.-P. Yap, L. D. Hughes, R. Park, P. S. Conti, *Mol. Pharm.* **2013**, *10*, 417–427.
- [4] Y. Shao, W. Liang, F. Kang, W. Yang, X. Ma, G. Li, S. Zong, K. Chen, J. Wang, *Amino Acids* **2014**, *46*, 2355–2364.
- [5] L. Chen, L. Du, M. Li, *Drug Des. Dev. Ther.* **2013**, *7*, 124–125.
- [6] J. M. Martínez, I. Prieto, M. J. Ramírez, C. Cueva, F. Alba, M. Ramírez, *Clin. Chem.* **1999**, *45*, 1797–1802.
- [7] I. Pérez, A. Varona, L. Blanco, J. Gil, F. Santaolalla, A. Zabala, A. M. Ibarguen, J. Irazusta, G. Larrinaga, *Head Neck* **2009**, *31*, 1335–1340.
- [8] L. Chen, W. Sun, J. Li, Z. Liu, Z. Ma, W. Zhang, L. Du, W. Xu, H. Fang, M. Li, *Org. Biomol. Chem.* **2013**, *11*, 378–382.
- [9] J. Li, L. Chen, W. Wu, W. Zhang, Z. Ma, Y. Cheng, L. Du, M. Li, *Anal. Chem.* **2014**, *86*, 2747–2751.
- [10] D. V. Hingorani, B. Yoo, A. S. Bernstein, M. D. Pagel, *Chem. Eur. J.* **2014**, *20*, 9840–9850.
- [11] K. R. Keshari, D. M. Wilson, *Chem. Soc. Rev.* **2014**, *43*, 1627–1659.
- [12] K. Golman, R. in't Zandt, M. Thaning, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 11270–11275.
- [13] F. A. Gallagher, M. I. Kettunen, D. E. Hu, P. R. Jensen, R. in't Zandt, M. Karlsson, A. Gisselsson, S. K. Nelson, T. H. Witney, S. E. Bohndiek, S. E. Hansson, T. Peitersen, M. H. Lerche, K. M. Brindle, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 19801–19806.
- [14] T. B. Rodrigues, E. M. Serrao, B. W. C. Kennedy, D.-E. Hu, M. I. Kettunen, K. M. Brindle, *Nat. Med.* **2013**, *20*, 93–97.
- [15] M. M. Chaumeil, P. E. Z. Larson, H. I. Yoshihara, O. M. Danforth, D. B. Vigneron, S. J. Nelson, R. O. Pieper, J. J. Phillips, S. M. Ronen, *Nat. Commun.* **2013**, *4*, 2429.
- [16] D. M. Wilson, R. E. Hurd, K. Keshari, M. Van Criekeing, A. P. Chen, S. J. Nelson, D. B. Vigneron, J. Kurhanewicz, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 5503–5507.
- [17] D. W. Christianson, W. N. Lipscomb, *Acc. Chem. Res.* **1989**, *22*, 62–69.
- [18] B. J. Campbell, Y.-C. Lin, R. V. Davis, E. Ballew, *Biochim. Biophys. Acta Enzymol. Biol. Oxid.* **1966**, *118*, 371–386.
- [19] R. Igić, S. Garber, M. Sekosan, R. A. Urbanska, R. Behnia, *Peptides* **2003**, *24*, 1237–1240.
- [20] P. Kugler, *Histochemistry* **1981**, *72*, 269–278.
- [21] S. Satoh, Y. Keida, Y. Konta, M. Maeda, Y. Matsumoto, M. Niwa, M. Kohsaka, *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* **1993**, *1163*, 234–242.
- [22] Phebestin inhibits also aminopeptidase A (APA,  $\text{IC}_{50} = 20 \mu\text{M}$ ) and aminopeptidase B (APB,  $\text{IC}_{50} = 20 \mu\text{M}$ ). However, the concentration of phebestin in this experiment was lower than their  $\text{IC}_{50}$  values, see: M. Nagai, F. Kojima, H. Naganawa, M. Hamada, T. Aoyagi, T. Takeuchi, *J. Antibiot.* **1997**, *50*, 82–84.

Received: October 9, 2015

Revised: November 9, 2015

Published online: December 21, 2015