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## Biotinyl-methyl 4-(amidomethyl) benzoate is a competitive inhibitor of human biotinidase

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### Abstract

Posttranslational modification of histones by biotinylation can be catalyzed by both biotinidase (BTD) and holocarboxylase synthetase (HCS). Biotinylation of histones is an important epigenetic mechanism to regulate gene expression, DNA repair, and chromatin remodeling. The role of BTD in histone biotinylation is somewhat ambiguous, given that BTD also catalyzes removal of the biotin tag from histones. Here, we sought to develop BTD inhibitors for future studies of the role of BTD in altering chromatin structure. We adopted an existing colorimetric BTD assay for use in a novel 96-well plate format to permit high-throughput screening of potential inhibitors. Biotin analogs were chemically synthesized and tested for their ability to inhibit human BTD. Seven of these compounds inhibited BTD by 26% to 80%. Biotinyl-methyl 4-(amidomethyl) benzoate had the largest effect on BTD, causing an 80% inhibition at 1 mM concentration. Enzyme kinetics studies were conducted to determine  $V_{max}$ ,  $K_m$ , and  $K_i$  for the seven inhibitors; kinetics were consistent with the hypothesis that biotinyl-methyl 4-(amidomethyl) benzoate and the other compounds acted by competitive inhibition of BTD. Finally, biotinyl-methyl 4-(amidomethyl) benzoate did not affect biotin transport in human cells, suggesting specificity in regard to biotin-related processes.

### Keywords

biotinidase; biotinyl-methyl 4-amidomethyl benzoate; histones; inhibitors

### 1. Introduction

Mammals contain two biotin-metabolizing enzymes, namely holocarboxylase synthetase (HCS) and biotinidase (BTD). HCS is a cytoplasmic, mitochondrial, and nuclear protein [1–3] whereas BTD is predominantly secreted into serum [4,5] but can also be found in the cell nucleus [2,4]. HCS catalyzes the covalent binding of biotin to a specific lysine residue in apocarboxylases to produce holocarboxylases [6]. The classical function of BTD is to recycle biotin by hydrolyzing the amide bond between biotin and lysine residues (biocytin) in breakdown products of holocarboxylases [7].

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Recently it was proposed that both BTD and HCS also catalyze the biotinylation of  $\epsilon$ -amino groups in specific lysine residues in histones [1,8–11]. Posttranslational modifications of histones have distinct functions in chromatin metabolism. For example, trimethylation of K4 in histone H3 is associated with transcriptional activation of genes, whereas dimethylation of K9 in histone H3 is associated with transcriptional silencing [12,13].

All five major classes of histones are targets for biotinylation in human cells [10]. While the physiological roles of histone biotinylation are under investigation, there is increasing evidence that histone biotinylation plays a role in DNA repair [14], and gene silencing and heterochromatin structures in human cells [15], and stress resistance and lifespan in *Drosophila* [16,17]. Biotinylation of histones is mediated by both HCS [1,16] and BTD [8], but evidence has been provided that HCS is the dominant histone-biotinyl ligase [16].

Biotinylation of histones is a reversible modification. Ballard et al. suggested that debiotinylation of histones might be mediated by BTD [18]. The regulation of BTD to favor debiotinylation of histones over biotinylation of histones by the same enzyme is unknown. A number of variables may regulate the catalytic activity of BTD. First, the availability of substrate might favor either biotinylation or debiotinylation of histones. For example, locally high concentrations of biocytin might shift the reaction equilibrium towards biotinylation of histones [8,19]. Second, proteins may interact with BTD at the chromatin level, favoring either biotinylation or debiotinylation of histones. Third, three alternatively spliced variants of BTD have been identified [20]. Theoretically, these variants may have unique functions with regard to histone biotinylation. Fourth, BTD possesses six glycosylation sites [21,22]; glycosylation of BTD might affect its cellular location [23].

Our long-term goal is to identify the roles of BTD in biotinylation and debiotinylation of histones. As a first step towards this goal, we generated a first generation of synthetic inhibitors of BTD, and we developed a 96-well plate assay for high-throughput screening of putative BTD inhibitors.

Previous studies have proposed using biotin, di-isopropylfluorophosphate, and thiol reagents such as *p*-chloromercuribenzoate as inhibitors of BTD [4]. These compounds are of limited use for the following two reasons. First, di-isopropylfluorophosphate and *p*-chloromercuribenzoate are general inhibitors of enzymes as opposed to being specific inhibitors of BTD. Second, simultaneous inhibition of BTD in both cytoplasm and nucleus make it impossible to link potential effects of low BTD activity to altered histone debiotinylation in the nucleus as opposed to impaired biotin recycling in the cytoplasm. The studies presented here lay the groundwork for the development of second-generation inhibitors that can be targeted directly to the nucleus to interfere with histone debiotinylation without affecting cytoplasmic events.

## 2. Materials and methods

### 2.1. BTD purification

Human plasma, which is known to contain high levels of BTD activity [4], served as a source of BTD in this study. Approximately 150 mL of human blood was collected through venipuncture into heparinized containers. This study was approved by the Institutional Review Board for Human Subjects at the University of Nebraska-Lincoln. Plasma was separated from blood cells by centrifugation [870 g for 30 min]. Previous studies indicated that BTD precipitates between 30% to 50% saturation with ammonium sulfate [24]. Thus, for all experiments described here plasma BTD was precipitated using 30% to 50% saturation with ammonium sulfate. Precipitated proteins, usually from 10 mL of plasma were re-suspended in 1.5 mL of potassium phosphate buffer (0.1 M, pH 6) and dialyzed for 24 to 36 hours against

three changes of 1 L of potassium phosphate buffer at 4°C. Dialyzed samples were subdivided into 1-mL aliquots and stored at -20°C. Please note that these crude preparations of BTD might contain proteins that affect analysis of  $V_{\max}$ ,  $K_m$ , and  $K_i$ . Individual aliquots were thawed and used as needed for BTD assays as described below. The amount of protein was quantified by using the Nanodrop 1000 spectrophotometer (Wilmington, DE). Plasma BTD activity is known to vary by about 25% among healthy individuals [25], explaining the sample-to-sample variation described below.

## 2.2. BTD inhibitors

Putative inhibitors of BTD were synthesized by conjugating biotin to various allylamines using carbodiimide as the coupling reagent. Briefly, equimolar amounts (0.21 mmol) of d-biotin (50 mg), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (39 mg), 4-(dimethylamino)pyridine (25 mg), allylamine derivatives (Table 1) were dissolved in 5 mL of *N,N*-dimethylformamide in an 8-mL vial. The solution was stirred under nitrogen atmosphere overnight. After the reaction was complete [checked by thin-layer chromatography and stained with iodine or phosphomolybdic acid solution], DMF was evaporated under vacuum. The crude product was column chromatographed on silica gel (Silica gel 60, 40–63  $\mu\text{m}$ , EMD Chemical Inc.; Gibbstown, NJ). The column was first washed with 50 mL dichloromethane and the product eluted with 150 mL dichloromethane:methanol (10:1, by volume). Solvents were removed by vacuum drying to obtain a white solid powder product at a 40% to 90% yield.  $^1\text{H}$  and  $^{13}\text{NMR}$  was used to verify the identity of the product.

## 2.3. BTD assay

BTD activity was measured as the rate of hydrolysis of *N*-biotinyl-4-aminobenzoic acid to release 4-aminobenzoic acid (PABA). The latter was quantified using *N*-1-naphthylethylenediamine dihydrochloride as described by Knappe et al. [26] and Backman-Gullers et al. [27], and modified by Nilsson & Ronge [28]. These protocols were adapted for microtiter plates as follows. In a 96-well microtiter plate, 500  $\mu\text{g}$  of partially purified BTD from plasma and 12  $\mu\text{L}$  of 10 mM putative BTD inhibitors (120 nmoles/well; 1.0 mM final concentration) were mixed with 88  $\mu\text{L}$  of 54 mM sodium phosphate buffer (pH 6.0), containing 1.08 mM disodium EDTA and 4.3 mM cysteamine hydrochloride (prepared fresh); samples were preincubated at 37°C for 60 min. Then, 10  $\mu\text{L}$  of 6mM *N*-(+)-biotinyl-PABA (60 nmoles/well; 0.5 mM final concentration) were added to each well and plates were incubated for 120 min at 37°C. Reactions were stopped by adding 30  $\mu\text{L}$  of 6 M hydrochloric acid and the following compounds were added at 3-min intervals at room temperature: 55  $\mu\text{L}$  water, 15  $\mu\text{L}$  of 14.5 mM sodium nitrite (prepared fresh), 15  $\mu\text{L}$  of 43.8 mM ammonium sulfamate, and 15  $\mu\text{L}$  of 3.86 mM *N*-1-naphthylethylenediamine dihydrochloride. Incubation was continued for 10 min. Precipitated proteins were removed by centrifugation (1260 g for 10 min) and the supernatant was transferred to a new plate and the absorbance was measured at 546 nm. Previous studies suggested that BTD activity is maximal at 37°C and pH 6.0 [24] and, thus, all tests were run under these conditions. One unit of BTD activity is defined as the amount of protein required to release 1 nanomole of PABA 120  $\text{min}^{-1}$  under the conditions of the assay.

## 2.4. Enzyme kinetics

$K_m$ ,  $V_{\max}$ , and  $K_i$  [29] were determined as follows. The concentration of inhibitors was kept constant (0.5 mM) in enzyme assays as described above, while the concentration of the substrate *N*-(+)-biotinyl-PABA was varied from 0.05 mM to 1 mM. The enzyme kinetics module of Sigmaplot 10.0 was used for calculations [30].

## 2.5. Biotin transport

Theoretically, the biotin analogs tested here might affect both biotin transport into human cells and BTB activity. Here, biotin transport was quantified using a physiological concentration of [<sup>3</sup>H]biotin (475 pM) in the presence or absence of putative BTB inhibitors (0.5 mM) as described [31]; the  $K_m$  of biotin transporters is in the low micromolar range [32]. Human Jurkat cells were used for biotin transport studies [33].

## 2.6. Statistical analysis

Heterogeneous variances were identified by using Bartlett's test, and data were log transformed where applicable [34]. Significance of differences was tested by one-way ANOVA. Fisher's Protected Least Significant Difference procedure was used for posthoc testing. StatView 5.0.1 (SAS Institute; Cary, NC) was used to perform all calculations. Differences were considered significant if  $P < 0.05$ . Data are expressed as mean  $\pm$  SD.

## 3. Results

### 3.1. Calibration and linearity of the BTB assay

BTB activity was dependent on substrate concentration, enzyme abundance and time. First, known amounts (0 to 40 nanomoles) of synthetic PABA were used to generate a standard curve (Fig. 1). This assay was linear up to 40 nanomoles PABA. Second, dependence of BTB activity on substrate concentration was quantified by incubating BTB with 0 (control), 0.1, 0.25, 0.5, and 0.75 mM of *N*-(+)-biotinyl-PABA per well in a 96-well plate. PABA released from *N*-(+)-biotinyl-PABA reached a plateau at 0.5 mM of *N*-(+)-biotinyl-PABA per well as judged by absorbance at 546 nm (Fig. 2); all subsequent reactions were carried out using 0.5 mM of *N*-(+)-biotinyl-PABA per well. Third, partially purified BTB from plasma was added in increasing concentration: 0, 0.1, 0.2, 0.4, 0.5, 0.75, and 1 mg of protein per well. The assay was linear up to a concentration of 1 mg of partially purified BTB per well (Fig. 3). Fourth, a time course was conducted to determine whether the hydrolysis of *N*-(+)-biotinyl-PABA was linear with time. BTB and *N*-(+)-biotinyl-PABA were incubated in assay buffer for up to 4 hours after the addition of 0.5 mM of *N*-(+)-biotinyl-PABA at 37°C. The hydrolysis of *N*-(+)-biotinyl-PABA was linear up to 4 hours (Fig. 4); all subsequent incubations with BTB were carried out for 2 hours following the addition of 60 nanomoles (0.5 mM final concentration/well) *N*-(+)-biotinyl-PABA unless otherwise noted.

### 3.2. Inhibitors of BTB

Seven out of the thirteen compounds tested here inhibited BTB. In contrast, the following compounds did not significantly affect BTB activity and were used as negative controls in selected experiments: biotinyl diphenylamide; biotinyl dibenzylamide; biotinyl (R)-1-(1-naphthyl)ethylamide; biotinyl 2,4,6-trimethylbenzenamide; biotinyl 3,4-dimethanilide; and biotinyl 4-amidobenzonitrile. These compounds had no meaningful effects on BTB activity even if concentrations as high as 0.5 mM were tested. The compounds tested here are all biotin analogs and it is highly unlikely that concentrations greater than 0.5 mM can be achieved in biological fluids in vivo [35]. Figure 5 depicts compound biotinyl 2,4,6-trimethylbenzenamide as a representative example.

In contrast, the following compounds inhibited BTB activity by more than 25%: biotinyl anilide; biotinyl allylamide; biotinyl *N*-methylanilide; biotinyl-methyl 4-(amidomethyl) benzoate; biotinyl 2-amido-pyridine; biotinyl 4-amidophenylboronic acid; and biotinyl benzylamide. Biotinyl-methyl 4-(amidomethyl) benzoate was the most effective compound of all the inhibitors tested. For example, if BTB was incubated with *N*-(+)-biotinyl-PABA in the presence of biotinyl methyl 4-(amidomethyl) benzoate for 30, 60, and 120 min, the hydrolysis

of N-(+)-biotinyl-PABA was 80%, 59%, and 30%, respectively, of inhibitor-free controls containing only DMSO. A greater percentage inhibition at earlier time points is an event frequently seen with competitive inhibitors [29]. Importantly, the percent inhibition of BTM increased if concentrations of inhibitors were increased, consistent with competitive inhibition of BTM by biotinyl methyl 4-(amidomethyl) benzoate (Fig. 7). For example, if BTM was incubated with N-(+)-biotinyl-PABA in the presence of 0.01 mM, 0.1 mM, and 1 mM biotinyl methyl 4-(amidomethyl) benzoate, the inhibition of hydrolysis of N-(+)-biotinyl-PABA was 16%, 50%, and 80%, respectively, compared to inhibitor-free controls containing only DMSO (Fig. 7).

Enzyme kinetics studies were conducted to confirm that the biotin analogs tested here decreased BTM activity by competitive inhibition. In these experiments we kept the concentrations of inhibitors constant (0 vs. 0.5 mM) and varied the concentrations of substrate, N-(+)-biotinyl-PABA (0.05 mM to 1 mM). For all the inhibitors tested, the percent inhibition of BTM decreased if the concentration of N-(+)-biotinyl-PABA was increased.  $K_m$  and  $V_{max}$  values were consistent with competitive inhibition (Table 2). The  $K_i$  value for biotinyl methyl 4-(amidomethyl) benzoate was smaller than  $K_i$  for all the other inhibitors, suggesting that this compound has a greater affinity for BTM than the other compounds tested.

Finally, we determined whether biotinyl methyl 4-(amidomethyl) benzoate affected cellular biotin transport, potentially producing artifacts in future studies of BTM-mediated chromatin biology at the cellular level. Biotin uptake into Jurkat cells was quantified in the presence of excess biotinyl methyl 4-(amidomethyl) benzoate, biotinyl anilide (specificity control) and DMSO (negative control). Biotinyl methyl 4-(amidomethyl) benzoate did not affect biotin uptake into Jurkat cells compared with DMSO controls ( $13 \pm 0.6$  vs.  $13 \pm 0.9$  amol  $\times$   $5 \times 10^{-6}$  cells  $\times$  30min $^{-1}$ ;  $P > 0.05$ ; n=3) whereas biotinyl anilide caused a significant decrease of biotin uptake ( $5.4 \pm 0.3$  amol  $\times$   $5 \times 10^{-6}$  cells  $\times$  30min $^{-1}$ ;  $P > 0.05$ ; n=3).

#### 4. Discussion

Previous studies of BTM depended on using time-consuming cuvette-based assays [26–28]; making high-throughput screening of BTM inhibitors a tedious task. Here, we present a 96-well plate assay that for the first time offers the means for high-throughput screening of BTM activity and putative BTM inhibitors.

Using this assay and an array of synthetic biotin derivatives, we succeeded in identifying seven putative BTM inhibitors. The most efficient compound was biotinyl-methyl 4-(amidomethyl) benzoate which inhibited BTM up to 80% at a concentration of 1 mM. Our kinetics studies suggest that biotinyl-methyl 4-(amidomethyl) benzoate (and other compounds tested here) act by reversible, competitive inhibition of BTM.

The inhibitors presented here are biotin derivatives and are likely to primarily affect proteins involved in biotin metabolism such as BTM, HCS, and biotin transporters, as opposed to affecting enzyme activity globally. Unspecific inhibitors such as *para*-hydroxymercuribenzoate, hydroxylamine, and mercaptoethanol do not offer the degree of specificity needed for cell biology studies [8]. For example, mercaptoethanol reduces sulfhydryl groups in enzymes, causing conformational changes that affect enzyme activity. Our studies on biotin transport provide evidence that some BTM inhibitors do not affect biotin uptake, suggesting specificity for BTM. We are currently in the process of investigating effects of BTM inhibitors on other histone biotinyl ligases such as HCS.

Please note, however, that even if an inhibitor is specific for BTM, it will abolish biological functions of this enzyme to the same extent in various cell compartments. For example, loss of BTM activity may result in biological changes that could either be a result of decreased

recycling of biotin from breakdown products of carboxylases in the cytoplasm [36], or of remodeling of chromatin through histone debiotinylation in the nucleus [2]. Our first-generation inhibitors of BTM do not overcome the problem of specifically targeting cytoplasmic versus nuclear BTM. A second generation of BTM inhibitors is currently being developed in our laboratory. The goal of these ongoing studies is to create an inhibitor that specifically targets nuclear BTM. Also, we will seek to modify these second generation inhibitors so that they do not undergo significant metabolism and loss of activity. Once these second-generation inhibitors become available, we hope to specifically characterize roles of BTM in chromatin remodeling.

## Abbreviations

BTM, biotinidase; DMSO, dimethyl sulfoxide; HCS, holocarboxylase synthetase; MCD, multiple carboxylase deficiency; PABA, para-aminobenzoic acid.

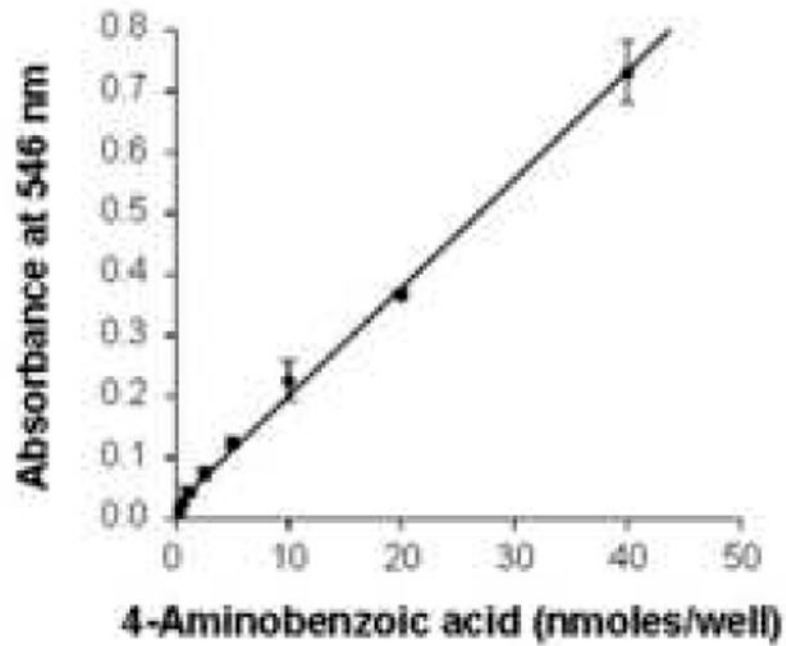
## Acknowledgment

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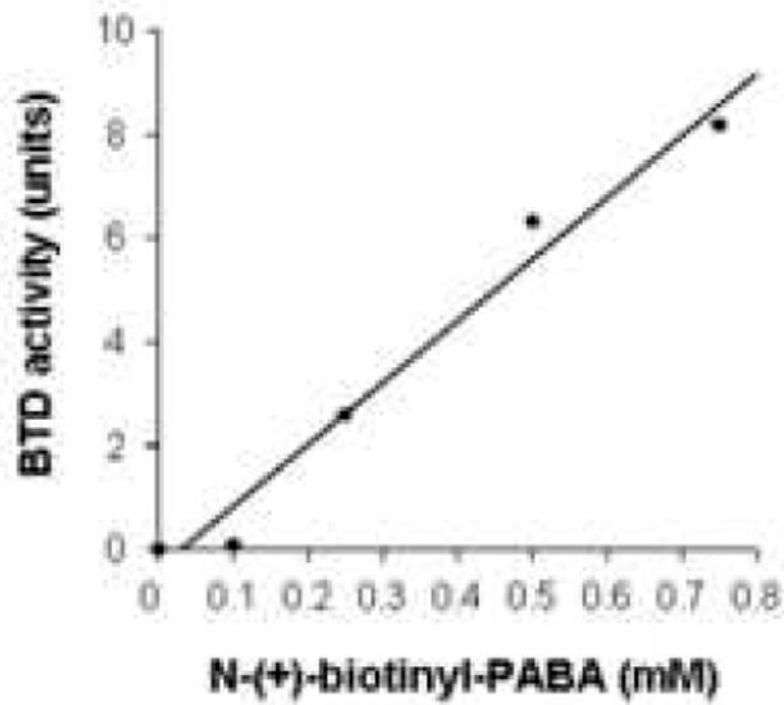
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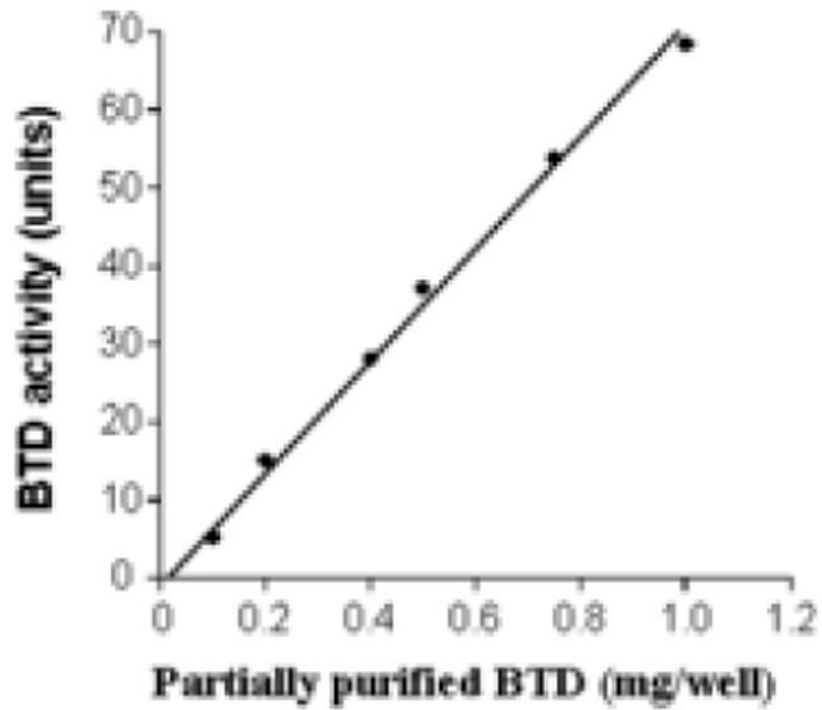


**Fig. 1.** Standard curve for colorimetric quantification of PABA. Absorbance of derivatives of PABA was measured at 546 nm. The standard deviations are too small to be visible at low amounts of PABA ( $y = 0.0013x + 0.021$ ;  $r = 0.998$ ).

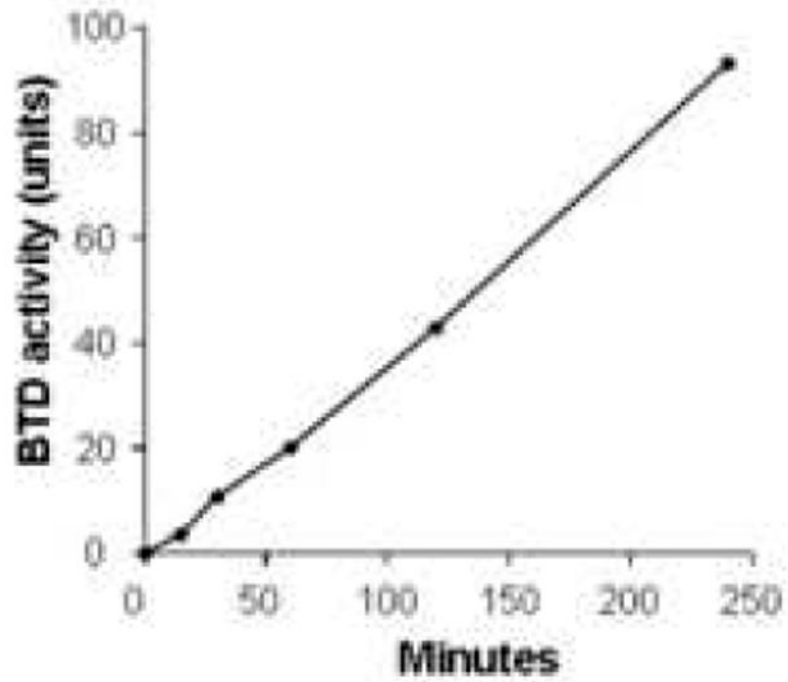




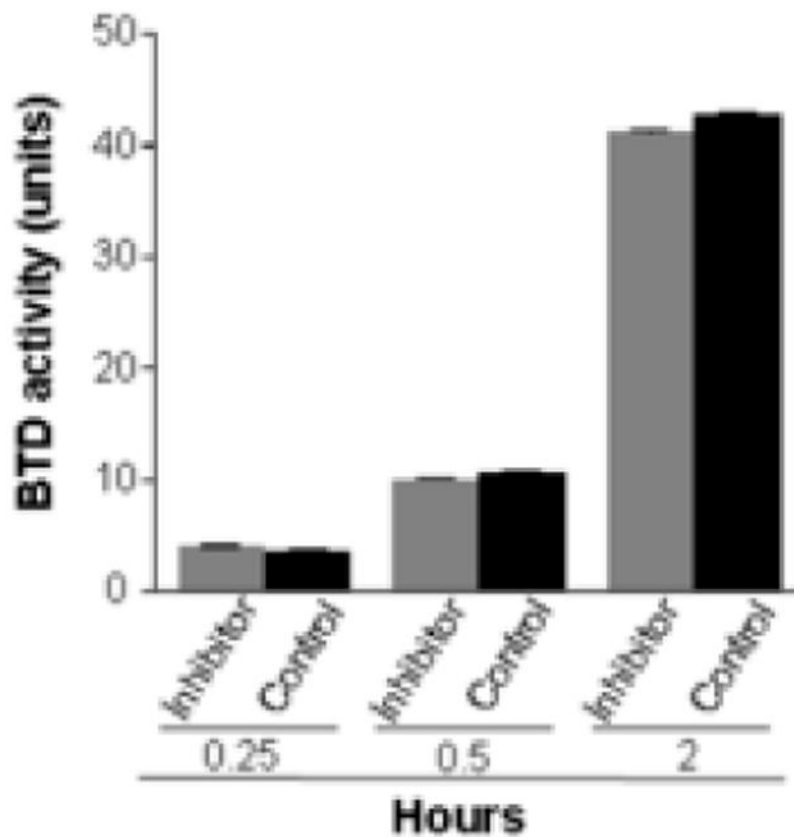
**Fig. 2.** BTD activity depends on the concentration of its substrate, N-(+)-biotinyl-4-amidobenzoic acid. BTD activity was monitored by the release of PABA from N-(+)-biotinyl-PABA. The standard deviations are too small to be visible ( $y = 0.11x - 1.19$ ;  $r = 0.989$ ).



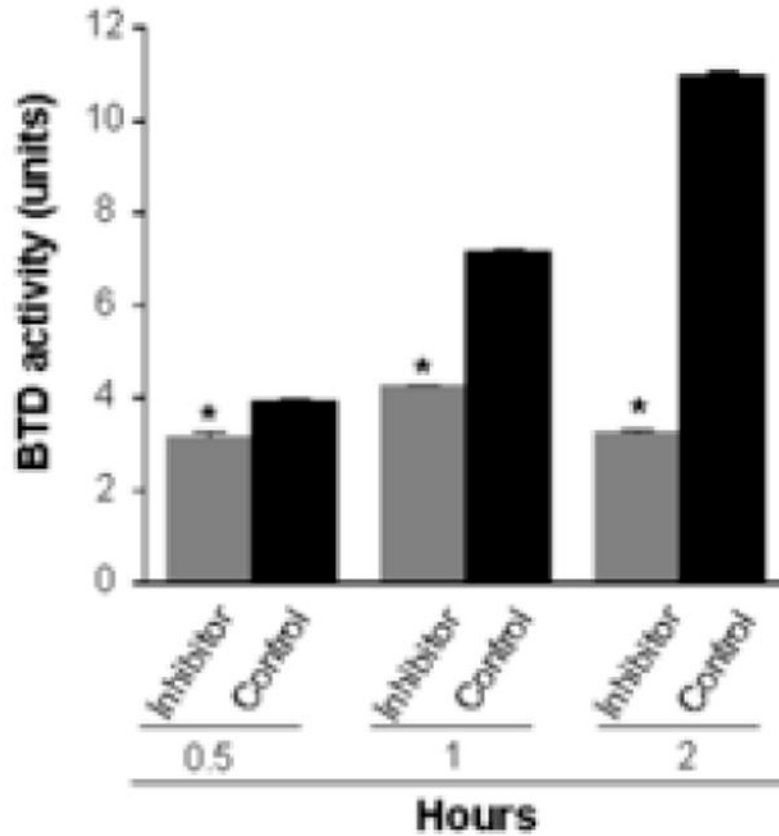
**Fig. 3.** Hydrolysis of N-(+)-biotinyl-4-amidobenzoic acid (60 nmoles/well) depends on the amount of partially purified BTD. The standard deviations are too small to be visible ( $y = 0.072x - 1.03$ ;  $r = 0.997$ ).



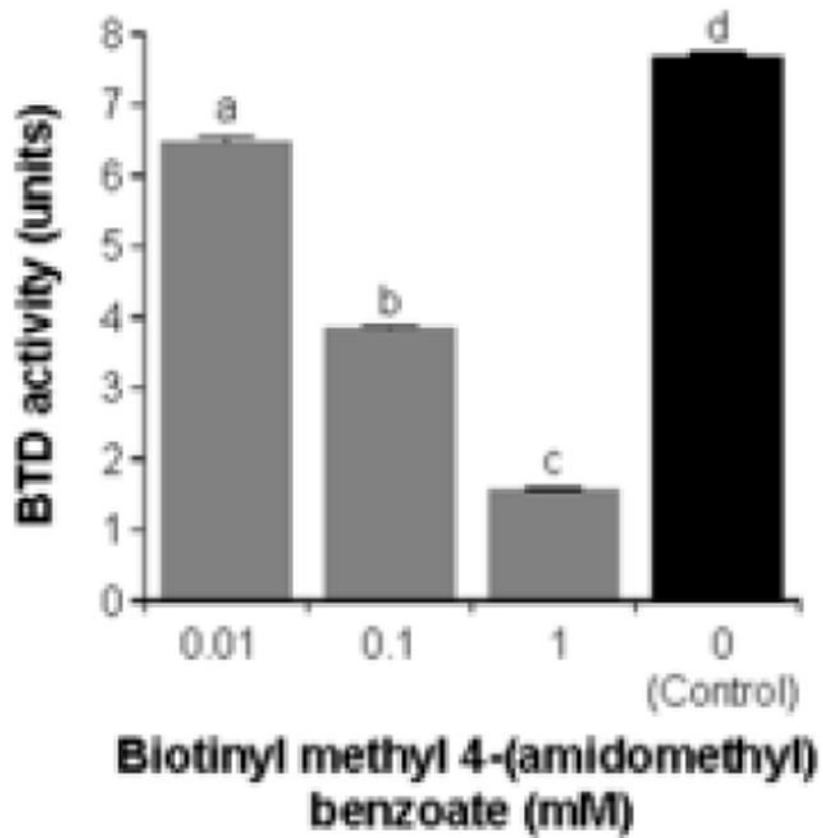
**Fig. 4.** Hydrolysis of N-(+)-biotinyl-4-amidobenzoic acid by partially purified BTD is linear for up to 4 hours. Values are means  $\pm$  SD (n = 6). The standard deviations are too small to be visible.



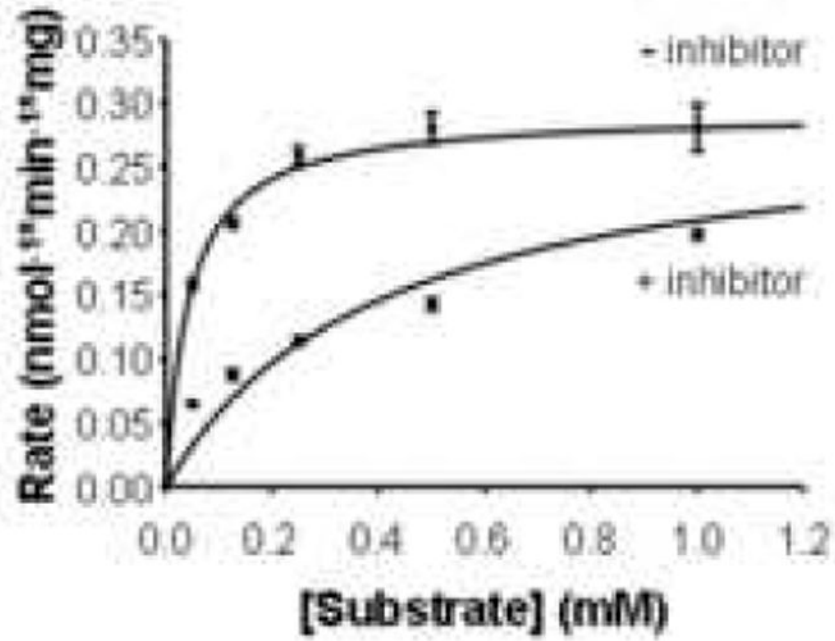
**Fig. 5.** Biotinyl 2,4,6-trimethylbenzenamide (1 mM) does not inhibit BTD. The effects of the treatment are not significantly different compared with inhibitor-free controls. The incubation times denote the hours of incubation of partially purified BTD, substrate, and inhibitor. Values are means  $\pm$  SD ( $n = 6$ ;  $P > 0.05$ ).



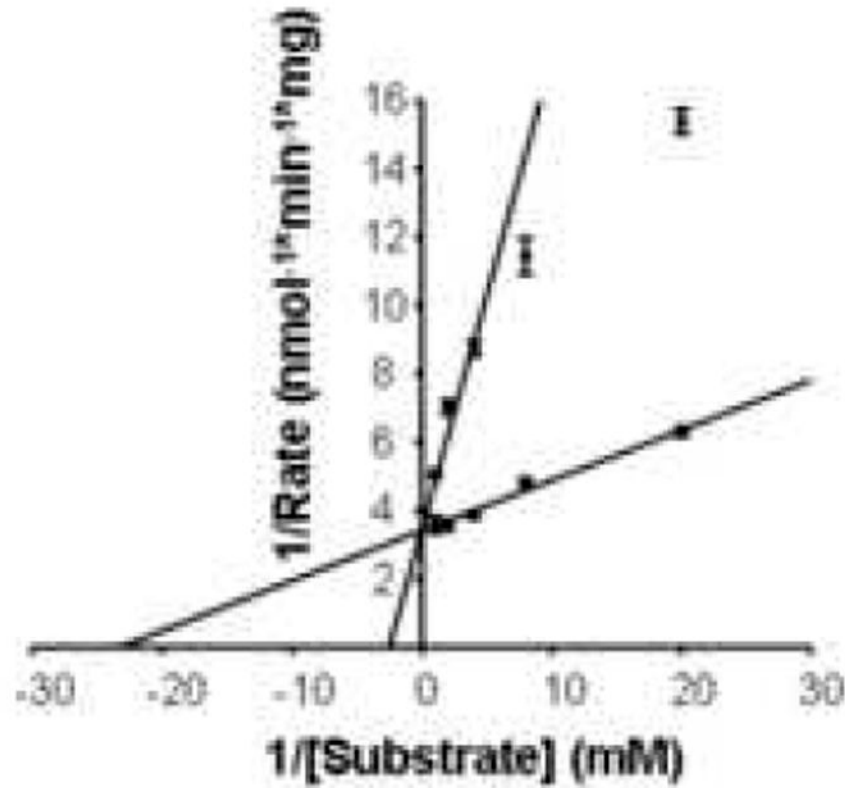
**Fig. 6.** Biotinyl-methyl 4-(amidomethyl) benzoate (1 mM) inhibits BTD. Values are means  $\pm$  SD (n = 6; \* $P$  < 0.05 compared with inhibitor-free control). The incubation times denote the hours of incubation of partially purified BTD, substrate, and inhibitor.



**Fig. 7.** Biotinyl-methyl 4-(amidomethyl) benzoate inhibits BTD activity in a concentration-dependent fashion. Values are means  $\pm$  SD ( $n = 6$ ). <sup>a,b,c,d</sup> Bars not sharing the same letter are significantly different from the inhibitor-free control ( $P < 0.05$ ).



**Fig. 8.** BTD activity is reduced in a substrate-concentration dependent manner in the presence or absence of 0.5 mM of the inhibitor biotinyl-methyl 4-(amidomethyl) benzoate.

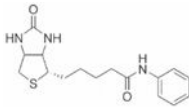
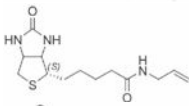
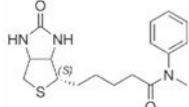
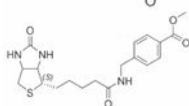
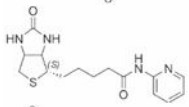
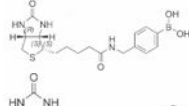
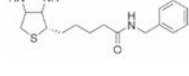


**Fig. 9.** Lineweaver-Burk plot demonstrating competitive inhibition of BTB by biotinyl methyl 4-(amidomethyl) benzoate.  $V_{\max} = 0.29 \pm 0.009$  nmol/min<sup>-1</sup>/mg<sup>-1</sup>;  $K_m = 0.04 \pm 0.007$  mM;  $K_i = 0.06 \pm 0.01$  mM;  $n = 3$ .



**Table 1**

## Inhibitors of BTD

Inhibitors	% inhibition <sup>a</sup>	Structure
Biotinyl anilide	55±0.1	
Biotinyl allylamide	37±0.09	
Biotinyl N-methylanilide	26±0.05	
Biotinyl methyl 4-(amidomethyl) benzoate	80±0.02	
Biotinyl 2-amido-pyridine	40±0.05	
Biotinyl 4-amidophenylboronic acid	53±0.02	
Biotinyl benzylamide	47±0.01	

<sup>a</sup>Percent inhibition compared with inhibitor-free control after 2 hours of incubation of BTD + inhibitor + substrate. Values are means ± SD (n = 6; *P* < 0.05 compared with inhibitor-free control).

**Table 2**Kinetics of BTD inhibitors<sup>a</sup>

Inhibitor name	Kinetic variables		
	$V_{\max}$	$K_m$	$K_i$
	$\text{nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$	mM	mM
Biotinyl methyl 4-(amidomethyl) benzoate	$0.29 \pm 0.001$	$0.04 \pm 0.0004$	$0.06 \pm 0.008$
Biotinyl anilide	$0.26 \pm 0.006$	$0.07 \pm 0.048$	$0.26 \pm 0.183$
Biotinyl 2-amido-pyridine	$0.28 \pm 0.002$	$0.01 \pm 0.003$	$0.27 \pm 0.011$
Biotinyl allylamide	$0.35 \pm 0.008$	$0.06 \pm 0.002$	$0.09 \pm 0.011$

<sup>a</sup>Data are means  $\pm$  S.D.