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Design, syntheses and evaluation of benzoylthioureas as urease inhibitors of agricultural interest

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Urea is some of the most used nitrogen fertilizers worldwide. However, occurrence of urea hydrolysis to ammonia and carbon dioxide on soil surface, catalysed by soil ureases, considerably reduces nitrogen availability to crops. In this study, we describe the design, synthesis and screening of sixty five benzoylthioureas (BTUs) for their ability to inhibit purified jack bean and soil ureases. BTUs were readily obtained in one pot, two steps synthesis with no need of cumbersome procedures for product purification. *In vitro* assays revealed BTUs **11**, **12**, **14**, **19-22** and **37** as the most active jack bean urease inhibitors. Such BTUs were found to be able to bind to both catalytic and allosteric sites of urease, acting therefore as mixed-type inhibitors. Out of 28 compounds that effectively inhibited soil ureases activity, BTUs **3**, **6**, **10**, **12**, **16**, **19** and **22** were determined to be more potent than the reference inhibitor *N*-(butyl)thiophosphoric triamide (**NBPT**; 40%). The other 22 BTUs were as potent as **NBPT** on soil ureases. The temperature-tolerance of BTUs, along with their ability to inhibit soil ureases, makes of this class of compounds potential additives for urea-based fertilizers.

Introduction

Urea is some of the nitrogen (N) fertilizers most widely used in agriculture because of its high N content (460 g kg^{-1}; N-urea). However, N loss by ammonia (NH_3) volatilization is a primary concern when urea is used in covering fertilization due to the activity of soil ureases on this fertilizer.[1] The average N losses via NH_3 volatilization in Europe was found to be 14% of total urea applied to soil[2] with these losses being higher in tropical climate areas.[3] Indeed, several field studies in Brazil reported average N losses ranging from 20% to 60%.[4-6] The N losses from NH_3 volatilization are also an environmental concern since NH_3 can directly or indirectly contribute to the increase of greenhouse gases in atmosphere and contamination of streams and lakes *via* eutrophication.[7] Several substances, both natural and synthetic ones, have been evaluated for their potential to inhibit urease as a strategy to increase urea efficiency in agriculture and mitigate environmental pollution.[7-10] Taking covering fertilization as an example, the partial inhibition of soil ureases allows for slowing down urea hydrolysis on soil surface increasing the chances of urea incorporation to the soil after rain or irrigation episodes.[11]

Heavy metal ions (Hg^{2+}, Cd^{2+}, Ag^+ etc), boron compounds (boric acid derivatives), organic molecules such as thiols, thiourea, hydroxyurea, acylhydroxamic acids, amides, benzothiazoles and esters of phosphoric acid, quinones and (poly)phenolic compounds have been recognized as urease inhibitors.[10,12,13] However, the best results in soil have been achieved using urea analogs, particularly the *N*-(butyl) thiophosphoric triamide (**NBPT**), which was found to bind to active site of ureases after its conversion to the corresponding *oxo*-derivative by soil microbiota.[14,15] However, **NBPT** tends to lose efficiency upon the time of storage and water exposure.[16,17] The **NBPT** efficiency is also reduced in acidic soils and under high temperatures,[17] conditions in which ureases have higher catalytic activity.[5] Therefore, the development of novel urease inhibitors of agricultural interest is mandatory to overcome the drawbacks presented by urease inhibitors currently on the market.

Thiourea derivatives are notable for a plethora of biological activities such as antimicrobial[18,19], anti-malarial[20], anticancer[21], antihypertensive[22] and as inhibitor of the HIV reverse transcriptase.[23] Moreover, the potential of N^2-substituted benzoylthioureas as urease inhibitors of clinical interest was reported recently.[24,25] Besides the pharmacological properties, thiourea and derivatives have being shown to possess herbicide[26,27] and insecticide[28] activities. Additionally, the ability of regulating plant growth exhibited by thiourea derivatives appears to be selective since such class of compounds is generally well tolerated by crops.[29,30]

Considering the role of thiourea as urease inhibitor and the biological activities of its derivatives, this work focused on the design and synthesis of 65 benzoylthioureas (BTUs) with potential to inhibit the ureolytic activity of ureases. The inhibitory urease properties were investigated in assays performed with purified jack bean and soil ureases. Kinetic experiments were carried used to address the mechanism of urease inhibition triggered by the best BTUs.

Experimental section

Analysis grade solvents were used without further treatment unless otherwise stated. Acetone was dried over molecular sieves 3Å for c.a. 6-18 h before use. Melting points were determined on a Microquímica MQAPF 302 hot plate apparatus. Infrared spectra (400-4000 cm^{-1}) were recorded as KBr discs on a Shimadzu FT-IR Model 8300 instrument. NMR spectra were obtained on a Bruker spectrometer Model Avance III operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C using a 5 mm broadband probe. NMR resonances were registered using CDCl$_3$ or DMSO-d_6 as solvents and TMS as internal standard. Chemical shifts (δ in ppm) were referenced to the residual solvent signals (CHCl$_3$ in CDCl$_3$ at δ 7.24; DMSO in DMSO-d_6 at δ 2.50). The splitting of proton resonances in the reported ^{1}H NMR spectra are defined as singlet (s), doublet (d), triplet (t), quartet (q) and complex pattern (m). Coupling constants (*J*) are reported in Hz.

Synthesis of benzoylthioureas (BTUs)

The N^2-substituted benzoylthioureas (BTUs) **1-65** were synthesized according to methodology described elsewhere[31], with modifications. Briefly, suitable benzoyl chloride (11 mmol) was added to ammonium thiocyanate (440 mmol L^{-1}) in acetone (25 mL). The reaction mixture was heated under reflux for 15 min and then cooled down to room temperature. An acetone solution of suitable amine (11 mol L^{-1}) was added to this mixture, which was stirred under reflux for further 30 min (for BTUs **4-65**) or under room temperature for 3 h (for BTUs **1-3**). The reaction mixture was then poured into crushed ice and the resulting mixture was vigorously stirred. Each obtained solid product was filtered off, washed with deionized water and then recrystallized from ethanol or ethanol in acetone.

Full data set for the novel BTUs are listed as follows. ***N*-(4-Hydroxyphenylcarbamothioyl)-3-methoxybenzamide (BTU 59):** Recrystallization from ethanol (74% yield). FT-IR (KBr, cm^{-1}): 3358 (amide N-H), 3014 (thiourea N-H), 1663 (C=O), 1271 (C=S). ^{1}H NMR (400 MHz, DMSO-d_6, ppm): 3.85 (s, 3H, OCH$_3$); 6.80 (d, *J* = 8.2 Hz, 2H); 7.21 (d, *J* = 8.2 Hz, 1H); 7.44 (m, 3H); 7.55 (m, 2H); 9.59 (s, 1H, OH); 11.49 (s, 1H, CONH); 12.42 (s, 1H, CSNH). ^{13}C NMR (DEPT-135 phase) (100 MHz, DMSO-d_6, ppm): 55.5(+) (OCH$_3$); 113.2(+); 115.1(+); 119.4(+); 121.0(+); 126.0(+); 129.4; 129.6(+); 133.5; 155.8; 159.0; 168.0 (C=O); 179.0 (C=S). ***N*-(4-Hydroxyphenylcarbamothioyl)-2-methoxybenzamide (BTU 64):** Recrystallization from ethanol (82% yield). FT-IR (KBr,

cm^{-1}): 3299 (amide N-H), 3025 (thiourea N-H), 1661 (C=O), 1254 (C=S). ^{1}H NMR (400 MHz, DMSO-d_6, ppm): 4.01 (s, 3H, OCH$_3$); 6.80 (d, *J* = 8.5 Hz, 2H); 7.16 (t, *J* = 7.5 Hz, 1H); 7.28 (d, *J* = 8.5 Hz, 1H); 7.46 (d, *J* = 8.5 Hz, 2H); 7.66 (t, *J* = 7.5 Hz, 1H); 7.93 (d, *J* = 7.5 Hz, 2H); 9.62 (s, 1H, OH); 11.15 (s, 1H, CONH); 12.35 (s, 1H, CSNH). ^{13}C NMR (DEPT 135 phase) (100 MHz, DMSO-d_6, p.p.m.): 56.7(+) (OCH$_3$); 112.8(+); 115.1(+); 119.5; 121.3(+); 125.8(+); 129.2; 131.2(+); 135.1(+); 155.9; 157.5; 165.2 (C=O); 177.8 (C=S). The data for previously reported BTUs to which spectral values have not been provided in the literature so far (NR; Tables 1 and 2) are included as supplementary information.

Thermogravimetric measurements

Thermogravimetric measurements were carried out in a Shimadzu TGA-50 Thermogravimetric Analyzer according to da Silva et al. (2013),[32] except that nitrogen gas flow (50 mL min^{-1}) was used instead of air flow. The weight change curves were recorded from 30 ^{o}C to 500 ^{o}C.

Urease inhibition assay

The screening for identifying potential urease inhibitors was done using the indophenol method.[33] Each benzoylthiourea (BTU) at final concentration of 500 μM was incubated in a medium reaction buffered with 20 mM phosphate (pH 7.4) and supplemented with 1 mM EDTA, 10 mM urea and 12.5 mU of *Canavalia ensiformis* (jack bean) type III urease (Sigma). Reactions were maintained at 25 °C for 10 to 15 min, followed by addition of 0.5 volume of 1% w/v phenol in 5 ppm sodium nitroprusside (SNP) and 0.7 volume of 0.5% w/v NaOH in 0.1% v/v NaOCl to interrupt enzyme activity. Reactions were then incubated at 50 °C for 5 min prior the measurement of absorbance at 630 nm to determine the amount of ammonium (NH$_4^+$) formed. Hydroxyurea (**HU**) and thiourea (**TU**) were used as references of urease inhibitors. Urease inhibition was determined in terms of percentage of NH$_4^+$ formed in BTU-containing reactions in relation to total urease activity in reactions devoid of inhibitor.

Kinetic assays with jack bean type III urease

The effect of BTUs synthesized on the kinetic parameters of jack bean type III urease was investigated using a procedure similar to that adopted for the *in vitro* screening, except that different concentrations of BTUs (250 to 500 μM) and urea (1 to 32 mM) were employed in the reaction media. Each BTU was used at concentrations necessary to inhibit the jack bean urease ureolytic activity in the range from 30 to 40%. Kinetic parameters of BTU-free and BTU-containing reactions were obtained using Hyper32 software. OriginPro 8 software was used to obtain Michaelis-Menten hyperbolas and Lineweaver-Burk plots to assess the mechanism by which the BTUs inhibit the ureolytic activity of urease.

Soil ureases activity assay

The effect of synthesized BTUs on the activity of soil ureases was assessed using the salicylate method.[34] Clayey dystrophic Red Latosol (oxisol) soil was collected from Brazilian Cerrado (19°28'01.2"S, 44°10'24.5"W). The physical features of the collected soil were 6% coarse sand, 4% fine sand, 12% silt, and 78% clay and chemical analyses showed pH 6.3, 10 mg dm^{-3} P$_{Mehlich-1}$, 129 mg dm^{-3} K, 4.4 cmol$_c$ dm^{-3} Ca, 0.9 cmol$_c$ dm^{-3} Mg, 0.1 cmol$_c$ dm^{-3} Al, 2.6 cmol$_c$ dm^{-3} H+Al, sum of bases of 5.6 cmol$_c$ dm^{-3}, 68% base saturation and organic matter of 2.5 dag kg^{-1}. Sieved soil (0.5 g; particles < 2 mm) were incubated with 72 mM urea in the presence or absence of each BTU (500 µM) at 37 °C for 1 h. The activity of soil ureases was interrupted by the addition of 1 M KCl in 10 mM HCl (5 mL). After 30 min incubation at 25 °C, a supernatant fraction was collected and added to a mixture constituted of 3.4% sodium salicylate, 2.5% sodium citrate, 2.5% sodium tartrate and 120 ppm SNP. Systems were incubated for further 15 min at 25 °C and under darkness. Then, 0.1 volume of 3.0% NaOH in 1.0% sodium hypochlorite was added to each system following incubation under darkness for 1 h at 25 °C and stirring (600 rpm). Spectrophotometric measurements were carried out at 660 nm to estimate NH$_4^+$ formed in the reaction media. The N-(butyl) thiophosphoric triamide (**NBPT**) was used as a reference of urease inhibitor active on soil ureases.

Statistical analysis

Data obtained from experiments carried out with soil were analyzed by one-way ANOVA followed by a Stepwise contrast test ($P < 0.001$) using the R software.

Results and discussion

Preparation of benzoylthioureas (BTUs)

The design of BTUs was based on thiourea (**TU**) structure, a known urease inhibitor.[12] Then, N^1,N^2-disubstituted thioureas bearing cores **A** and **B** constituted of aliphatic, aromatic, heteroaromatic, benzylic and homobenzylic groups were synthesized to check if the structural modifications improved the ability of thiourea to inhibit ureases (Fig. 1).
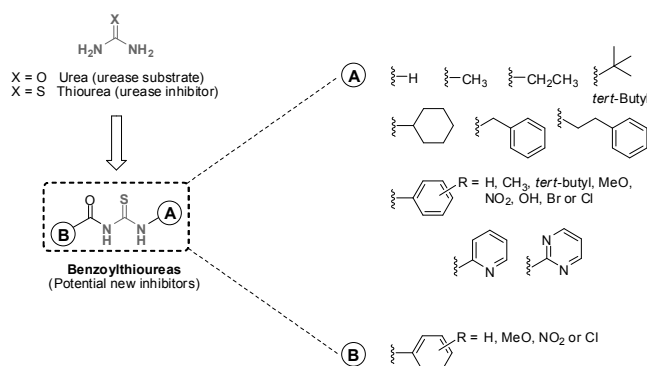
Fig. 1. Strategy used for the design of urease inhibitors of agricultural interest based on benzoylthiourea moiety.

The BTUs **2-65** (Tables 1 and 2) were prepared by reacting primary amines with *in situ*-produced benzoyl isothiocyanate.[31] Compounds **4-65** (Fig. 1) were obtained after 30 min under reflux while adducts **2** and **3** were formed after 3 h at room temperature under stirring. The BTUs synthesized were obtained in moderate to high yields (Tables 1 and 2). The BTU **1** was synthesized in a similar manner, except that NH$_4$OH was used instead of an amine. Measured melting points and/or spectroscopic data (IR; ^{1}H and ^{13}C NMR) for the BTUs herein synthesized were compared to those reported in the literature, when available (Tables 1 and 2). The structures of the novel BTUs **59** and **64** were confirmed by spectroscopic techniques. Strong C=O stretching absorption and weak N-H stretching were observed for all compounds in IR spectra between 1700-1660 cm^{-1} and 3419-3011 cm^{-1}, respectively. A complex set of bands, characteristic of the thioamide moiety were also observed in accordance to data reported elsewhere.[35] The C=S stretching was observed between 1291-1236 cm^{-1}.

Table 1. Synthetic approach for obtaining benzoylthioureas based on the use of benzoyl chloride as a start material.

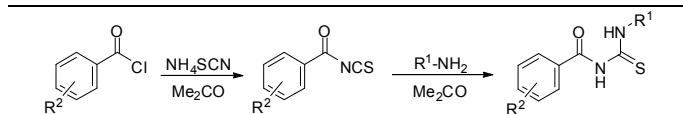| BTU	R^1	Yield (%)^a	M.p. (°C)	Ref.
1	H	50	170	43
2	CH$_3$	38	147	44
3	CH$_2$CH$_3$	58	125	NR
4	C$_4$H$_9$	58	124	44
5	C$_6$H$_{11}$	90	64	45
6	C$_6$H$_5$	79	146	46
7	C$_6$H$_5$CH$_2$	80	121	47
8	C$_6$H$_5$(CH$_2$)$_2$	86	105	48
9	4-OMe-C$_6$H$_4$	92	145	49
10	4-OH-C$_6$H$_4$	96	160	50
11	4-(CH$_3$)-C$_6$H$_4$	94	156	49
12	4-(C$_4$H$_9$)-C$_6$H$_4$	79	114	NR
13	4-NO$_2$-C$_6$H$_4$	93	179	51
14	4-Cl-C$_6$H$_4$	97	136	46
15	4-Br-C$_6$H$_4$	85	144	50
16	3-OMe-C$_6$H$_4$	87	105	49
17	3-OH-C$_6$H$_4$	70	184	52
18	3-NO$_2$-C$_6$H$_4$	89	158	51
19	3-Cl-C$_6$H$_4$	99	125	49
20	3-Br-C$_6$H$_4$	85	129	53
21	2-OMe-C$_6$H$_4$	92	150	51
22	2-NO$_2$-C$_6$H$_4$	64	146	51
23	2-Cl-C$_6$H$_4$	74	142	46
24	2-Br-C$_6$H$_4$	86	134	54
25	2-pyridinyl	71	137	41
26	2-pyrimidinyl	73	176	55

^{a}Values correspond to the overall yield of two-step reactions. **M.p.**, melting point as compared to that reported elsewhere (if available). **NR**, not yet reported so far.

All detected ^{1}H and ^{13}C NMR peaks were consistent with the presence of the corresponding aliphatic or aromatic group in

BTUs structure. The CON-*H* and CSN-*H* protons peaks were observed as broad singlets between δ_H 8.69-12.35 and δ_H 9.85-13.60, respectively. The ^{13}C NMR peak between δ_C 176.8-182.1 was assigned to C=S carbon present in each BTU structure. Furthermore, carbonyl signal was observed in δ_C 164.9-168.6 range, which is the typical region for C=O of benzoylthioureas.[31]

Table 2. Synthetic approach for obtaining benzoylthioureas based on the use of substituted-benzoyl chlorides as start materials.

BTU	R^1	R^2	Yield (%)^a	M.p.	Ref.
27	C$_6$H$_5$CH$_2$	4-NO$_2$-C$_6$H$_4$	49	143	56
28	4-MeO-C$_6$H$_4$	4-NO$_2$-C$_6$H$_4$	76	149	57
29	4-OH-C$_6$H$_4$	4-NO$_2$-C$_6$H$_4$	68	172	NR
30	4-NO$_2$-C$_6$H$_4$	4-NO$_2$-C$_6$H$_4$	95	192	58
31	4-Cl-C$_6$H$_4$	4-NO$_2$-C$_6$H$_4$	83	173	NR
32	C$_6$H$_5$	3-NO$_2$-C$_6$H$_4$	73	149	NR
33	C$_6$H$_5$CH$_2$	3-NO$_2$-C$_6$H$_4$	73	104	NR
34	4-MeO-C$_6$H$_4$	3-NO$_2$-C$_6$H$_4$	77	156	NR
35	4-OH-C$_6$H$_4$	3-NO$_2$-C$_6$H$_4$	81	195	NR
36	4-NO$_2$-C$_6$H$_4$	3-NO$_2$-C$_6$H$_4$	89	173	NR
37	4-Cl-C$_6$H$_4$	3-NO$_2$-C$_6$H$_4$	89	155	NR
38	C$_6$H$_5$	2-NO$_2$-C$_6$H$_4$	82	172	NR
39	C$_6$H$_5$CH$_2$	2-NO$_2$-C$_6$H$_4$	60	156	NR
40	4-MeO-C$_6$H$_4$	2-NO$_2$-C$_6$H$_4$	76	179	NR
41	4-OH-C$_6$H$_4$	2-NO$_2$-C$_6$H$_4$	73	215	59
42	4-NO$_2$-C$_6$H$_4$	2-NO$_2$-C$_6$H$_4$	43	216	NR
43	4-Cl-C$_6$H$_4$	2-NO$_2$-C$_6$H$_4$	77	202	NR
44	C$_6$H$_5$	4-Cl-C$_6$H$_4$	99	144	NR
45	C$_6$H$_5$CH$_2$	4-Cl-C$_6$H$_4$	77	120	60
46	4-MeO-C$_6$H$_4$	4-Cl-C$_6$H$_4$	99	146	61
47	4-OH-C$_6$H$_4$	4-Cl-C$_6$H$_4$	99	186	NR
48	C$_6$H$_5$	3-Cl-C$_6$H$_4$	68	135	NR
49	C$_6$H$_5$CH$_2$	3-Cl-C$_6$H$_4$	72	126	NR
50	4-MeO-C$_6$H$_4$	3-Cl-C$_6$H$_4$	79	128	NR
51	4-OH-C$_6$H$_4$	3-Cl-C$_6$H$_4$	75	182	NR
52	4-OH-C$_6$H$_4$	2-Cl-C$_6$H$_4$	78	198	NR
53	C$_6$H$_5$	4-OMe-C$_6$H$_4$	98	123	NR
54	C$_6$H$_5$CH$_2$	4-OMe-C$_6$H$_4$	94	126	NR
55	4-OH-C$_6$H$_4$	4-OMe-C$_6$H$_4$	81	170	NR
56	4-NO$_2$-C$_6$H$_4$	4-OMe-C$_6$H$_4$	95	180	NR
57	C$_6$H$_5$	3-OMe-C$_6$H$_4$	82	103	NR
58	C$_6$H$_5$CH$_2$	3-OMe-C$_6$H$_4$	76	85	NR
59	4-OH-C$_6$H$_4$	3-OMe-C$_6$H$_4$	94	177	NR
60	4-Cl-C$_6$H$_4$	3-OMe-C$_6$H$_4$	96	123	NR
61	C$_6$H$_5$	2-OMe-C$_6$H$_4$	78	139	NR
62	C$_6$H$_5$CH$_2$	2-OMe-C$_6$H$_4$	69	96	NR
63	4-MeO-C$_6$H$_4$	2-OMe-C$_6$H$_4$	95	116	NR
64	4-OH-C$_6$H$_4$	2-OMe-C$_6$H$_4$	82	184	NR
65	4-Cl-C$_6$H$_4$	2-OMe-C$_6$H$_4$	76	145	NR

^{a}Values correspond to the overall yield of two-step reactions. **M.p.**, melting point as compared to that reported elsewhere (if available). **NR**, not yet reported so far.

The BTUs derived from benzoyl chloride and aliphatic amines or ammonia (**1** to **4**) were obtained in 38-60% yield (Table 1). Benzoyl chloride and aromatic amines led to the formation of BTUs **5** to **26** in yields ranging from 64% to 99% (Table 1). Substituted-benzoyl chlorides in the presence of a variety of aromatic amines or benzylamine provided the remainder BTUs

(**27-65**; Table 2) in 40% to 99% yield. Interesting features of the herein reported BTUs preparation include **(i)** the starting materials are commercially available, **(ii)** products are obtained in one-pot reaction carried out in only two steps with no need of further purification of benzoyl isothiocyanates formed as intermediates, **(iii)** products are easily purified from recrystallization and **(iv)** products are solid, facilitating storage and transportation. These BTU properties are particularly interesting when compared to those of *N*-(butyl) thiophosphoric triamide (**NBPT**), a urease proinhibitor commonly used as additive in urea-based fertilizers. Differently from the BTUs, **NBPT** is difficult to handle because it is obtained as a waxy and sticky material.[17]

Inhibitory effect of BTUs on jack bean urease activity

An *in vitro* screening was carried out with 65 BTUs in the presence of purified jack bean type III urease and urea to investigate the potential of these synthesized compounds to inhibit the ureolytic activity of ureases. Hydroxyurea (**HU**) and thiourea (**TU**), commonly used in *in vitro* screenings[36-39], were employed as references of urease inhibitors for comparison purposes.

Fifty one out of 65 BTUs at 500 μM inhibited at different extents urease activity in medium reactions containing 10 mM urea (Table 3). Compounds **3**, **4**, **6**, **10-12**, **14**, **16**, **19-22**, **37**, **45**, **55**, **57**, **59** and **60** were the most active BTUs toward jack bean urease as attested by percentages of enzyme inhibition equal or higher than 40% (Table 3). Notably, the effect of BTU **14** on jack bean urease was comparable to that of the reference inhibitor **HU** and 3.2-fold higher than that of the inhibitor **TU**. On the other hand, inhibition lower than 10% categorized the BTUs **15**, **30**, **36** and **41** as poor inhibitors of jack bean urease.

In fact, *in vitro* assays with jack bean ureases have been widely used to assess the potential of several substances as urease inhibitors of clinical and agricultural interests.[10] Eighteen BTUs were found to be the most active against jack bean type III urease because they inhibited the ureolytic activity of such enzyme by 40-75% (Table 3). Among them, those (12 BTUs) derived from non-substituted benzoyl chloride inhibited the urease by up to 75% while the ones derived from substituted benzoyl chloride (except for **37**) caused a maximum of 50% enzyme inhibition. Indeed, compound **37** that bears *p*-Cl phenyl and *m*-NO$_2$ phenyl groups as cores **A** and **B**, respectively, inhibited the urease activity by 67% (Fig. 1). As for non-substituted benzoyl chloride derived BTUs, the presence of phenyl ring bearing *p*-tert*-butyl, *o*-NO$_2$ or (*m*- or *p*-)Cl or (*m*- or *p*-)Br groups as the core **A** seems to contribute to the effectiveness of BTUs against jack bean urease. These results corroborate those obtained by Rauf et al. (2013)[24] in which the presence of halogen substituents in phenyl ring at core **A** improved the BTU urease activity. Additionally, previously reported BTUs bearing *p*-OCOC$_2$H$_5$ phenyl as core **A** and *p*-

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OCH$_3$ or m,p-diOCH$_3$ groups as core **B** were determined as the most active urease inhibitor among the compounds tested.[25]

Table 3. Inhibitory activity of synthesized benzoylthioureas (0.5 mM) toward jack bean urease in reactions containing 10 mM urea.

BTU	Urease inhibition (%)	BTU	Urease inhibition (%)
1	32.0	35	NA
2	11.2	36	8.7
3	43.8	37	66.9
4	44.0	38	NA
5	24.2	39	29.2
6	47.4	40	NA
7	27.0	41	3.8
8	17.7	42	NA
9	31.7	43	NA
10	40.7	44	17.7
11	51.7	45	47.6
12	64.2	46	NA
13	27.3	47	NA
14	73.9	48	13.8
15	6.4	49	37.5
16	49.1	50	NA
17	24.1	51	28.4
18	32.2	52	26.5
19	60.0	53	33.0
20	65.2	54	20.1
21	51.0	55	39.1
22	57.2	56	17.6
23	33.4	57	43.5
24	19.7	58	30.6
25	NA	59	41.6
26	NA	60	49.3
27	17.9	61	22.3
28	NA	62	12.6
29	NA	63	15.2
30	5.1	64	29.6
31	28.3	65	10.8
32	NA	HU	73.6
33	16.3	TU	22.6
34	NA		

NA, non-active with respect to the ability to inhibit urease activity when tested at our experimental conditions. Hydroxyurea (**HU**) and thiourea (**TU**) were used as references of urease inhibitor.

### Effect of BTUs on kinetic parameters of jack bean type III urease

Assays using fixed concentrations of BTUs in reactions containing urea in the range of 1 - 32 mM were performed to expand the knowledge on the type of enzyme inhibition triggered by the most active BTUs synthesized. The BTUs that caused urease inhibition by over 50%, namely **11**, **12**, **14**, **19-22** and **37** (Table 3), were then selected for the studies of mechanism of urease inhibition. Reactions free of inhibitor were carried out whenever a BTU would be tested since slight changes in reaction conditions may affect the kinetic parameters. A V_o *versus* urea concentration plot obtained from data of assays with **11** is shown in Figure 2 to exemplify the

Michaelian behavior of urease catalysis. Similar behavior was observed in assays performed with the other seven BTUs. The average concentration of urea (K_M or K_S) that allows for urease velocity reaching 0.5 V_{max} in reactions buffered with phosphate (pH 7.4) was 4.2 ± 0.3 mM and the average V_{max} for urease was found to be 10.1 ± 0.3 μmol NH$_4^+$ min^{-1} mg^{-1} prot. The Lineweaver-Burk plots obtained (Fig. 2) indicate that all the BTUs tested function as mixed-type inhibitors toward urease, as the lines intersect one with another in the second quadrant. Mixed-type inhibitors are known to be able to bind to both the free enzyme (forming EI complex) and enzyme-substrate complex (forming ESI complex), therefore being associated to two equilibrium dissociation constants (K_i for the former and K_i' for the latter complexes). In fact, the equilibrium dissociation constants related to complexes former among urease, urea and each BTU individually (K_i values) were found to be higher than the equilibrium dissociation constants for complexes formed between urease, urea and the corresponding BTU (K_i' values) (Table 4). These results point out that the affinity of the BTUs object of this study for the urease active site is higher than that of allosteric site(s). The BTU **12** was the least potent with respect to the binding to either the active site or allosteric site(s) of urease. Some unsymmetrically-substituted N,N'-diaryl thiourea, compounds structurally related to BTUs, were recently described as mixed inhibitors of jack bean urease.[38]

Table 4. Effect of benzoylthioureas (BTUs) on jack bean urease kinetics. Urea (1 to 32 mM) was incubated for 5 min with jack bean Type III urease in the absence or presence of BTU (250 to 500 μM). The K_M (or K_S) and V_{max} values determined were, respectively, 4.2 ± 0.3 mM and 10.1 ± 0.3 μmol NH$_4^+$ min^{-1} mg^{-1} protein.

BTU	$^a K_i$ (μM)	$^a K_i$' (μM)
11	283.7 ± 25.8	631.4 ± 65.1
12	$1,776.6 \pm 670.0$	$5,170.8 \pm 524.0$
14	166.9 ± 66.0	380.2 ± 83.2
19	396.7 ± 11.1	$1,325.4 \pm 130.2$
20	475.2 ± 166.0	824.4 ± 169.9
21	795.6 ± 90.4	$1,211.9 \pm 117.1$
22	308.7 ± 91.8	$1,257.5 \pm 99.8$
37	416.9 ± 100.1	$1,349.9 \pm 379.6$

$^a K_i$ corresponds to the equilibrium dissociation constant for urease-BTU complex while K_i' is the equilibrium dissociation constant for urease-urea-BTU complex. Values are the mean ± standard deviation.
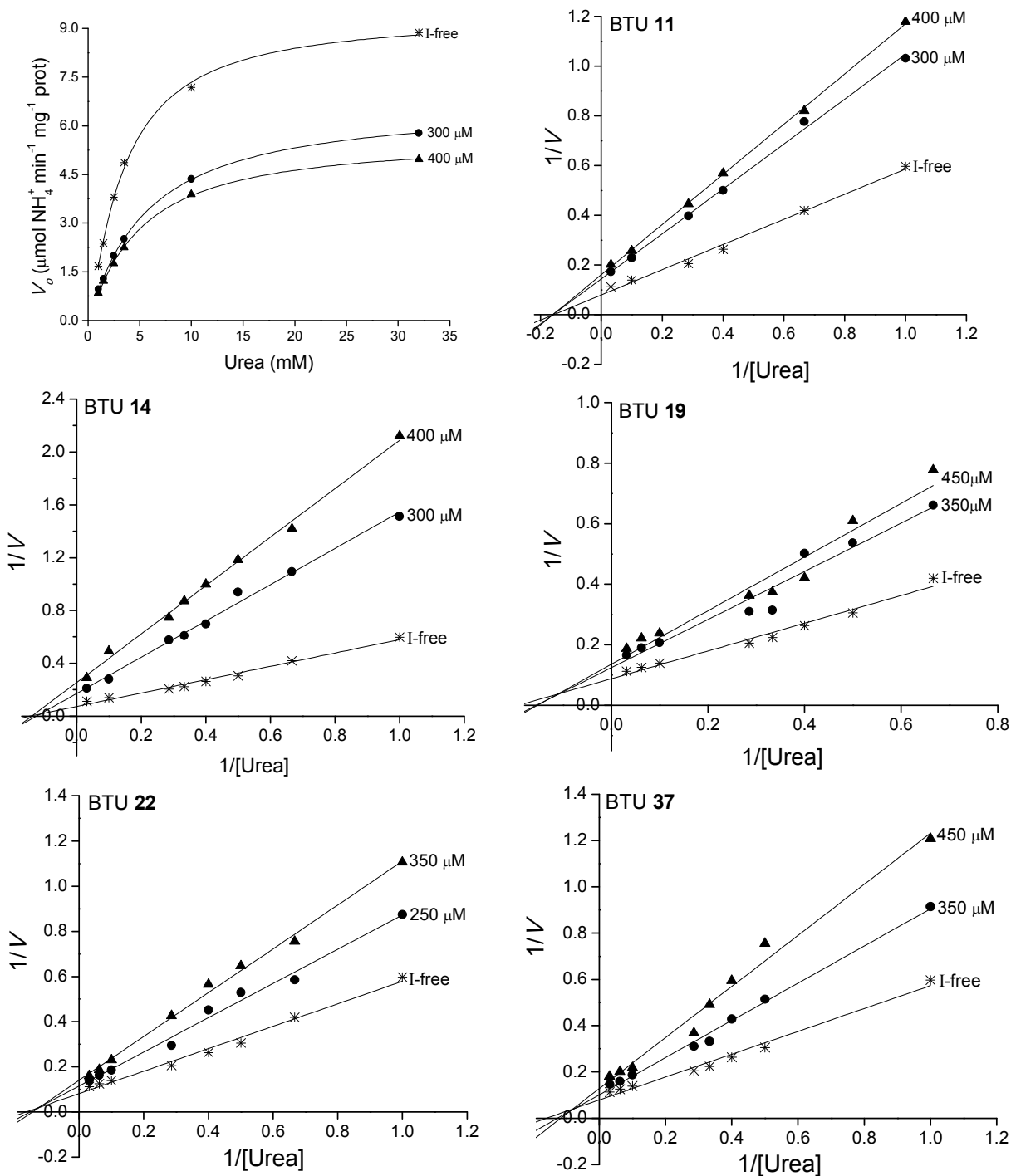
Fig. 2. Representative Michaelis-Menten hyperbola and Lineweaver-Burk plots for jack bean urease in the presence of the benzoylthioureas (BTUs). Increasing concentrations of urea were incubated for 5 min with jack bean Type III urease in the absence of inhibitor (I-free) or presence of BTU at indicated concentrations. A V_o *versus* urea concentration plot obtained from data of assays with **11** is shown to exemplify the Michaelian behavior of urea catalysis.

Journal Name

ARTICLE

Inhibition of soil ureases by BTUs

The results obtained from *in vitro* assays encouraged us to further investigate the potential of BTUs as urease inhibitors of agricultural interest against a soil used for crop production in Brazilian Cerrado. For these experiments, *N*-(butyl) thiophosphoric triamide (**NBPT**), an additive in urea-based fertilizers, was used as a reference of urease inhibitor.

Fig. 3 presents the results for 28 BTUs, found to inhibit the activity of soil ureases by at least 15%. Analysis of variance (one-way ANOVA) followed by Stepwise contrast test allowed to separate the BTUs in two groups of inhibitors in which the first one included the compounds more potent than **NBPT** (BTUs **3**, **6**, **10**, **12**, **16**, **19** and **22**; $P < 0.001$) and the second one comprised 21 compounds found to be as potent as **NBPT** (Fig. 3). Other 19 BTUs inhibited soil ureases in the range from 5% to 10% while compounds **7**, **8**, **13**, **23**, **28**, **36**, **40**, **46**, **47**, **50**, **58**-**65** failed to inhibit the formation of NH_4^+ catalyzed by soil ureases.

Although jack bean urease shares high homology of amino acid sequence with ureases of other organisms[12,40], contrasting results were obtained when comparing *in vitro* findings with those obtained from soil. For instance, while BTUs **26**, **32**, **34** and **38** where inactive in *in vitro* assays (Table 3), they were as effective as **NBPT** to inhibit soil ureases (Fig. 3). Then, it becomes evident that results from *in vitro* assays *per se* are not enough to select potential urease inhibitors of agricultural interest. However, assays performed with purified urease are important tools to pursue a better comprehension on the mechanism of action of a certain inhibitor as well as the structural features critical for the design of more potent inhibitors. Moreover, the complex physicochemical and biological nature of soil matrix may culminate in the metabolization of xenobiotic substances as is the case of synthetic urease inhibitors. Chemical transformations triggered by soil microbiota may result in loss- or gain-of-function of a certain xenobiotic.
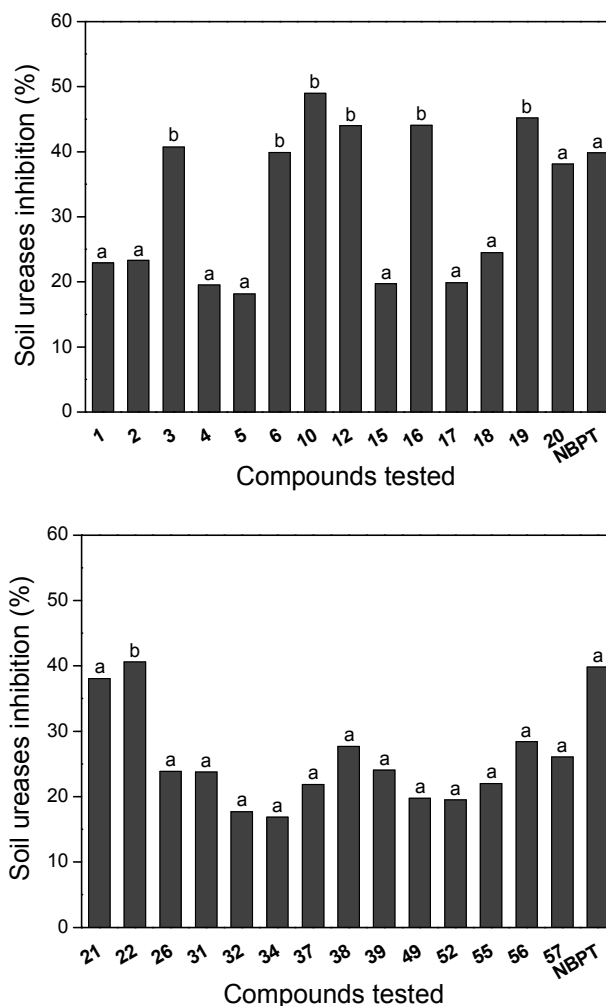

Fig. 3. Benzoylthiourea-triggered inhibition of soil ureases. Amended soil (0.5 g) was incubated with urea (72 mM) in the presence or absence of compounds-test at 0.5 mM. Results are representative of independent experiments, each done in quadruplicate or quintuplicate. Standard errors were lower than 11%. NBPT, reference soil urease inhibitor. Distinct letters indicate significant difference ($P < 0.001$) according to variance analysis (one-way ANOVA) followed by Stepwise contrast test.

Thermal stability of BTUs

The BTUs that clustered into the group of the best soil urease inhibitors (**3**, **6**, **10**, **12**, **16**, **19** and **22**) were then selected for assessing their thermal stability. The thermogravimetric curves clearly show that all BTUs tested are more thermal stable than **NBPT** (Fig. 4). The event of mass loss for compounds **10** (most

stable BTU) and **22** (less stable BTU) begins at 210 °C and 166 °C, respectively, while NBPT starts to lose mass at 151 °C (Fig. 4). The other BTUs (**3**, **6**, **12**, **16** and **19**) show a weight loss at temperature range from 180 °C to 195 °C. Other BTUs are reported to be resistant to heat.[25,41,42]
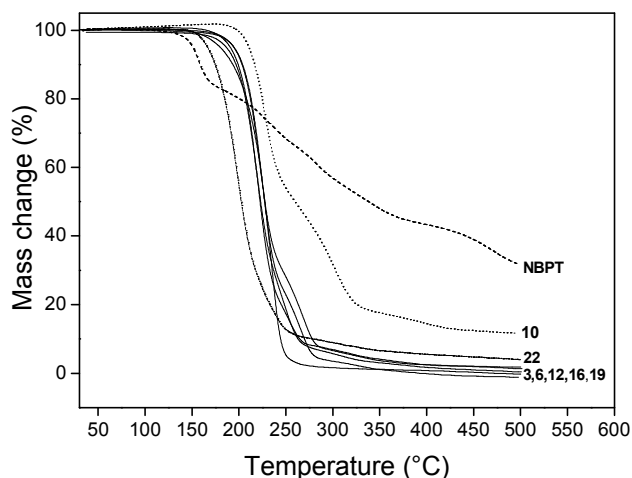
Fig. 4. Thermogravimetric behavior of the most promising soil urease benzoylthiourea inhibitors. **NBPT** was used as reference of soil urease inhibitor.

Conclusions

The design, synthesis and biological evaluation of 65 benzoylthioureas (BTUs) allow for the *in vitro* identification of eight inhibitors disclosed to exhibit mixed-type mechanism of urease inhibition. Screening on tropical soil revealed 28 BTUs as promising soil urease inhibitors, being compounds **3**, **6**, **10**, **12**, **16**, **19** and **22** the most active BTUs. The extent of urease inhibition *in vitro* is not always correlated to the results obtained for soil, requiring careful addressing: (**i**) the former allows for understanding the structure-activity relationship for the design of more potent inhibitors and the mechanism of action of such molecules as well; (**ii**) the latter is important to verify the behavior of a urease inhibitor-candidate toward a variety of soil ureases. Overall, this work demonstrated for the first time with basis on soil experiments the great potential of some benzoylthioureas as urease inhibitors of agricultural interest.

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Notes and references

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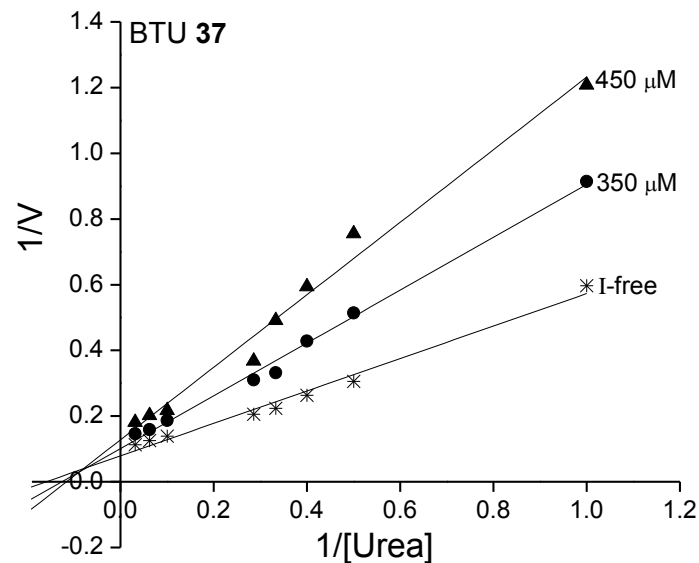†These authors contributed equally to this work.
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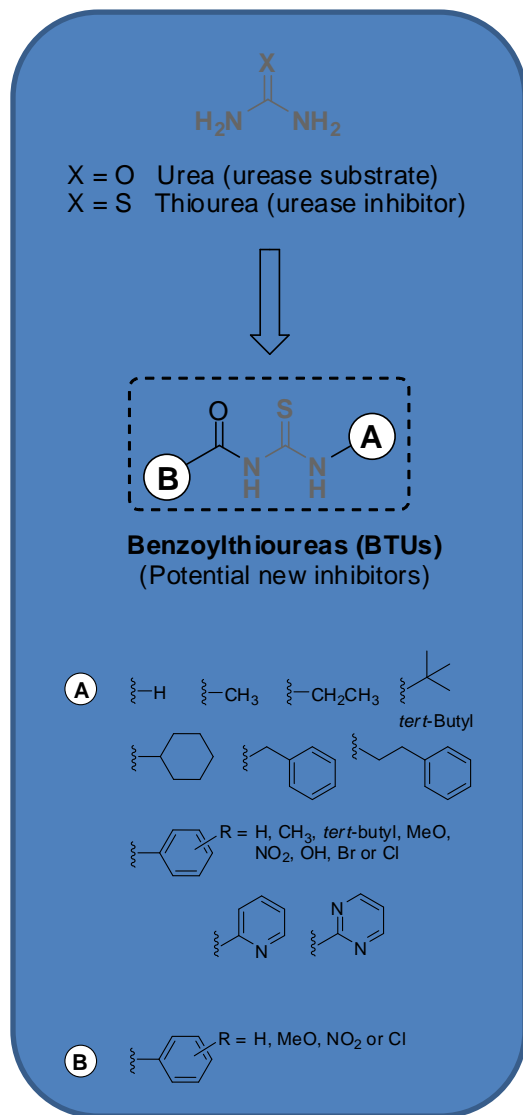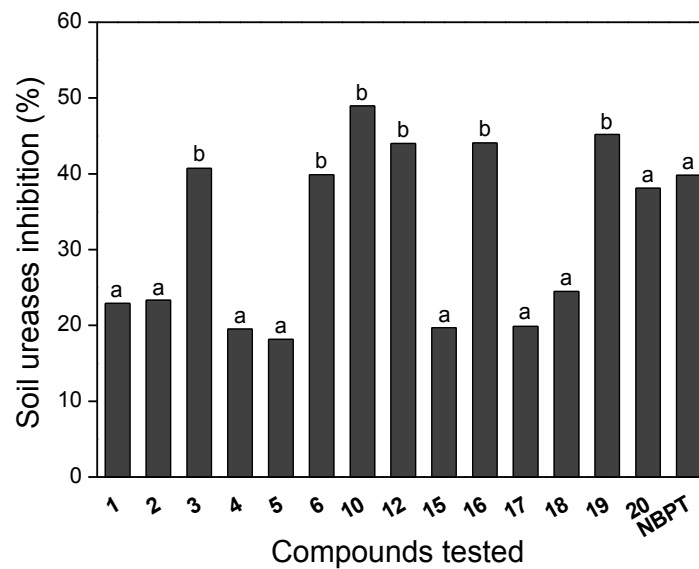
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Mechanism of urease inhibition

Inhibitors of agricultural interest