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Silica-supported fluoroboric acid (HBF_4–SiO_2) catalyzed highly productive synthesis of thiomorpholides as activators of L-asparaginase as well as the antioxidant agent

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ABSTRACT

An efficient solvent-free procedure for the synthesis of thiomorpholides in the presence of a catalytic amount of solid-supported fluoroboric acid (HBF_4–SiO_2) is described. The advantages of this method are high yields, short reaction times, ease of product isolation, low cost, and the catalyst can be recycled for a number of times without significant loss of activity. Three thiomorpholides possessing electron-donating group (**4c**, **4g**, and **4h**) were exhibiting excellent stimulatory activities against *Erwinia carotovora* L-asparaginase. The most potent activator, compound **4h** displayed the following kinetic parameters, K_m = 75 μM and V_{max} = 1000 μmol mg^{-1} min^{-1} and K_A = 0.985 μM. Furthermore, these compounds (**4g**, **4h**, **4c**, **4f**, **4a**, and **4d**) have also shown promising 2,2′-diphenyl-1-picrylhydrazyl (DPPH) reducing antioxidant activity (21–36%) at 1 mM concentration as compared to standard butylated hydroxyl anisole (72% at 1 mM).

1. Introduction

Thiomorpholides are of importance in medicinal chemistry[1] due to their biological activity, for example, against bacterial infection, as fungicides, herbicides, and antiulcerative agents.[2] Apart from these applications, thioamides are known to be versatile intermediates in organic synthesis,[3] particularly in the field of peptide chemistry[4] as well as building blocks for the synthesis of five- and six-membered heterocycles.[5] A traditional approach to their synthesis is the Willgerodt–Kindler reaction which has found only limited applications, because of the high reaction temperatures and long reaction periods required, and the low to moderate yields obtained.[6] Recently, the synthesis of thiomorpholide has been carried out using ionic liquid[7] and microwave.[8] However, some of the above-mentioned limitations are sill associated with both methods such as high temperature,[9,10] long reaction time,[11] expensive and large amount of catalyst.[12] To the best of our knowledge, uptil now the synthesis of thiomorpholides using the solid-supported catalyst as well as temperature lower than 100 °C has not been reported so far.

The toxic and volatile nature of many organic solvents, particularly chlorinated hydrocarbons that are widely used in organic synthesis have posed a serious threat to the environment. Consequently, methods that successfully minimize their use are the focus of much attention.[13,14] In recent years, the use of catalysts immobilized on solid supports has received considerable attention.[15–19] Such catalysts not only simplify the purification process but also help in preventing the release of reaction residues into the environment. Thus, the solvent-less condition along with supported catalyst provides a protocol for achieving environment friendly organic synthesis.

L-Asparaginase (L-asparagine amino hydrolase, E.C.3.5.1.1) catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia.[20–24] L-asparaginase from *Escherichia coli* and *Erwinia chrysanthemii* were used as chemotherapeutics in Acute Lymphoblastic Leukemia (ALL) for the past three decades.[25] The L-asparaginases from *E. coli* and *Erwinia carotovora* possess different immunological specificities and offer an important alternative therapy if a patient becomes hypersensitive to one of the enzyme.[26] The comparison of two enzymes leads to the conclusion that *E. coli*-asparaginase can be recommended for first-line therapy, reserving *Erwinia*-asparaginase for allergic patients, because most patients allergic to the former are not immediately allergic to the latter.[27] Enzyme activation by thiol group or thio group is not an uncommon event in enzymology. It was reported that thiol compounds such as *N*-acetyl cysteine and glutathione, in a fixed concentration potentate

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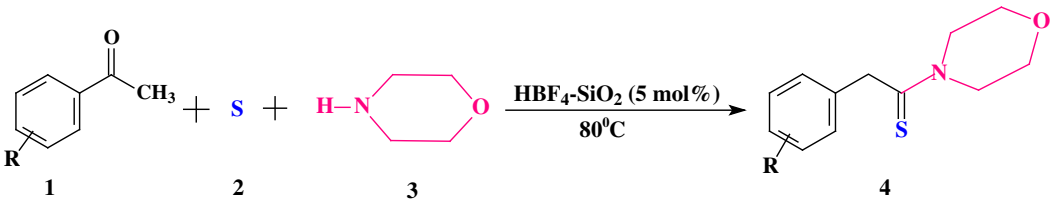


1 2 3 4

Scheme 1.

the activity of asparaginase from *Cylindrocarpon obtusisporum* MB-10,[28] *Pseudomonas stutzeri* MB-405,[29] and *E. carotovora*.[30] For instance, catalytic activities of cysteine protease from *Schistosoma mansoni*,[31] N^{ω}-phosphoarginine hydrolase from rat liver,[32] reticulocytic protein kinase,[33] glyoxylase,[34] the family C G-protein coupled receptors, that is, the metotropic glutamate receptors were profoundly accelerated by the addition of natural thiol compound.[35] The molecular mechanism behind the activation effects of enzymes by thiol compounds or thio group has been due to the redox changes.[36] *Erwinia* asparaginase activation considered because of the presence and involvement of free thio group in the enzyme catalysis.[37]

As reactive oxidative species (ROS) are involved in the pathogenesis of many diseases, finding efficient antioxidants as therapeutic agents have been one of the hottest areas in biomedicine. However, only relatively trivial success has been attained in antioxidant-based drug discovery over this period. According to the MDL Drug Data Report (MDDR) database, only two antioxidant drugs have been launched (i.e., idebenone and edaravone) in the past 30 years. Thus, it seems that it is urgent to improve the translationally efficiency of antioxidant research.

Free radical induced oxidative stress is linked to the majority of metabolic disorders such as cancers, inflammation, Parkinson's diseases, atherosclerosis, Alzheimer diseases, premature aging, and stroke.[38] The free radicals include superoxide anions, hydrogen peroxide, peroxyl radicals, reactive hydroxyl radicals, nitric oxide, and peroxynitrite anions, causing oxidative damage to cell components. Diseases caused by oxidative damage are prevented by antioxidant reagents that react with free radicals and chelating catalytic metals.[39,40]

In this communication, we wish to report simple and efficient method for the synthesis of thiomorpholides from various aryl alkyl ketones with sulfur and morpholine using solid-supported fluoroboric acid (HBF_4–SiO_2) and evaluated for their effect on kinetic parameters of *E. carotovora* asparaginase and antioxidant activity (Scheme 1).

2. Results and discussion

2.1. Chemistry

As part of our program aiming at developing new, selective, and environmental friendly methodologies for the preparation of biologically active medicinal scaffolds using HBF_4–SiO_2 as a catalyst,[41] we wish to describe herein a highly effective protocol for the synthesis of thiomorpholides from the reaction of various aryl alkyl ketones with sulfur and morpholine under solvent-free conditions (Scheme 1).

Initially, a systematic study was carried out for catalytic evaluation of HBF_4–SiO_2 as a catalyst for the reaction of acetophenone (1 mmol) with sulfur (1.2 mmol) and morpholine (1 mmol) under various conditions (Table 1). The reaction was very slow with low yield in the absence of catalyst at room temperature (Table 1, entry 1) as well as 80 °C (Table 1, entry 2). Next, we optimized the quantity of the HBF_4–SiO_2 at 80 °C under solvent-free conditions (Table 1, entries 3–9) and it was observed that the use of just 5 mol % of catalyst is sufficient to produce an excellent yield of the product (Table 1, entry 7). Whereas more than 5 mol % of the catalyst did not improve the results (Table 1, entries 8 and 9). Inferior results were obtained in the presence of solvents (Table 1, entries 10–12).

Table 1
Reaction of acetophenone with sulfur and morpholine under various conditions

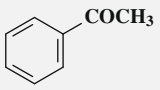
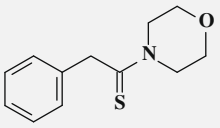
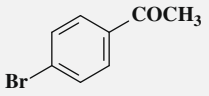
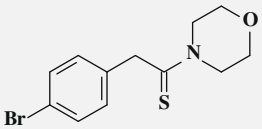
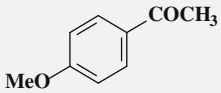
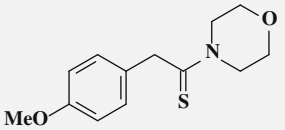
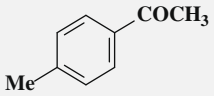
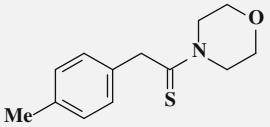
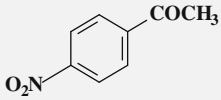
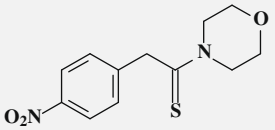
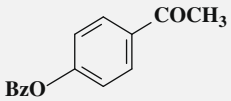
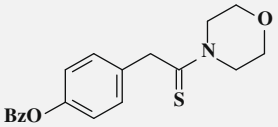
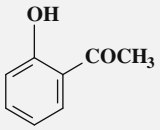
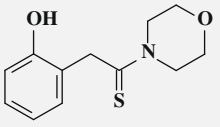
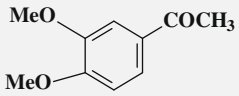
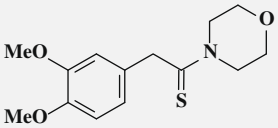
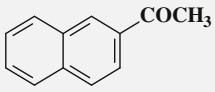
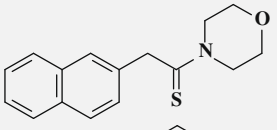
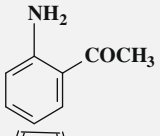
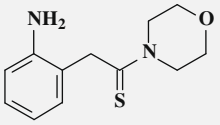
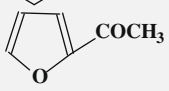
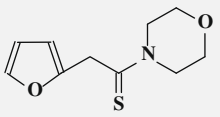
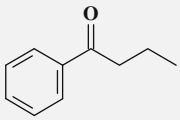
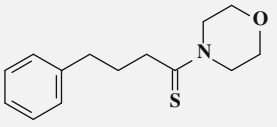
Entry	Solvent	Catalyst (mol %)	Temp (°C)	Time [h]/min	Yield (%)
1	Neat	—	rt	[48]	2
2	Neat	—	80	[30]	35
3	Neat	HBF_4–SiO_2 (**1**)	80	[3]	60
4	Neat	HBF_4–SiO_2 (**2**)	80	[2]	65
5	Neat	HBF_4–SiO_2 (**3**)	80	90	75
6	Neat	HBF_4–SiO_2 (**4**)	80	60	85
7	Neat	HBF_4–SiO_2 (**5**)	80	45	95
8	Neat	HBF_4–SiO_2 (**7**)	80	45	95
9	Neat	HBF_4–SiO_2 (**10**)	80	45	95
10	CH_2Cl_2	HBF_4–SiO_2 (**5**)	80	[2]	60
11	THF	HBF_4–SiO_2 (**5**)	80	[2]	55
12	$CHCl_3$	HBF_4–SiO_2 (**5**)	80	[2]	70

To establish the generality of this protocol, various aryl alkyl ketones were reacted with sulfur and morpholine in the presence of a catalytic amount of HBF_4–SiO_2 (5 mol %) at 80 °C under solvent-free conditions. The results were represented (Table 2). Both electron-rich and electron-deficient acetophenones reacted well with sulfur and morpholine to give thioamides in excellent yields. Bulky and sterically hindered substrates such as 2-hydroxyacetophenone, 2-acetylnaphthalene, and 2-aminoacetophenone reacted smoothly under similar conditions to produce the corresponding thioamides (Table 2, entries g, i, and j). In case of halogenated acetophenones, no side products were observed arising from nucleophilic displacement of halogen by morpholine under these conditions (Table 2, entry b). Another substrate such as butyrophenone also gave the corresponding thioamide in 89% yield (Table 2, entry l). In most of the cases, the products were obtained as solids.

In the classical Willgerodt–Kindler reaction, morpholine was generally used in excess solvent as well as reactant.[6,8] The use of excess ionic liquid as a solvent minimized the quantity of morpholine in the reaction.[7] Therefore, in this respect the present methodology using solvent-free condition is superior. Furthermore, under the classical conditions, the reactions require high temperature, longer reaction time (8–12 h) and low yields of products.[6,8] Yields are improved in case of method using ionic liquid.[7] However, longer reaction time (3–6 h) and drastic reaction conditions (110 °C) are the limitations associated with this green protocol. It is important to note that the present solid-supported method is better in comparison with the reported methods considering reaction time (0.75- 3.5 h), mild reaction condition (80 °C), and excellent yields (82–95%).

The advantage of the use of a heterogeneous catalyst for this transformation is the ease of catalyst/substrate separation. In our process, when the catalytic reaction was completed, HBF_4–SiO_2 may be recovered conveniently from the reaction mixture and used further for the next cycle without activation, only through filtration and subsequent washing with ethyl acetate. Efforts were made to examine the reusability of HBF_4–SiO_2 by using acetophe-

Table 2
Preparation of thiomorpholine using HBF_4–SiO_2 catalyst

Entry	Arylalkyl ketone (**1**)	Thiomorpholine[a] (**4**)	Yield[b] (%)	Time [h]/min
a			95	45
b			92	[2.2]
c			87	[2.0]
d			90	[2.2]
e			87	[3.0]
f			92	[2.5]
g			91	[2.1]
h			88	[3.5]
i			82	[3.0]
j			93	[2.0]
k			87	[2.5]
l			89	[2.6]

[a] All products were characterized by ^1H NMR, IR, and mass spectroscopy.
[b] Yield refers to pure product after column chromatography.

none (1 mmol) with sulfur (1.2 mmol) and morpholine (1 mmol) under solvent-free conditions at 80 °C as a model substrate and the results are presented (Fig. 1). It is observed that, with the increasing number of cycles of the reaction, the catalytic activity of the HBF_4–SiO_2 decreases, and it was nearly lost after 5th cycle.

2.2. Biological evaluation

The L-asparaginase was purified from *E. carotovora* by using single-step chromatography. The final recovery of enzyme was revealed as 68% with approximate 78% final purity (data not

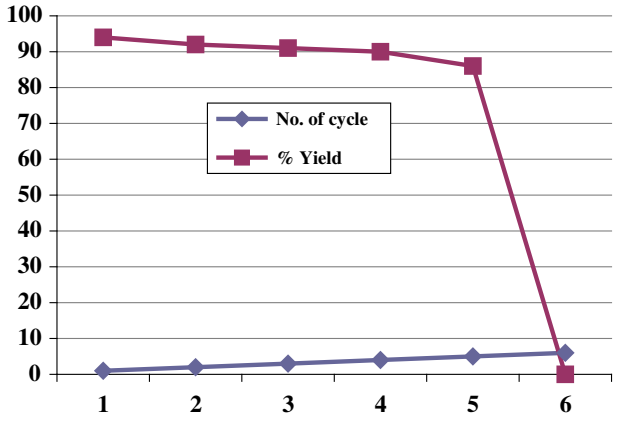


Figure 1. Effect of catalytic cycles on product yield.

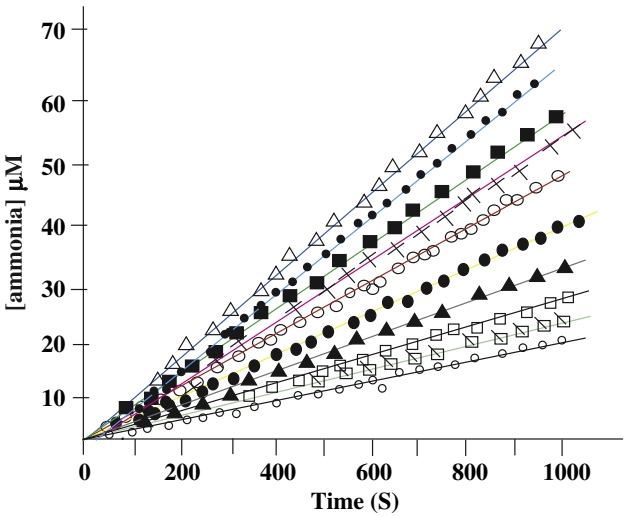


Figure 2. L-Asparaginase catalyzed production of L-aspartate and ammonia in the presence of increasing amounts of thiomorpholides (0–100 μM); closed triangles, 0 μM; closed circles, **4b** (50 μM); open circles, **4d** (50 μM); crosses, **4j** (50 μM); closed squares, **4g** (50 μM); small closed circles, **4c** (50 μM); open triangles, **4h** (50 μM). All are represented by solid lines, representing the best fitted lines, showing increased activity in its presence. While, lower dashed lines, representing decreased activity than control. Open squares, **4a** (50 μM); squares with lines, **4k** (50 μM); small open circles, **4e** (50 μM).

shown). The enzyme showed typical Michaelis–Menten kinetics at substrate concentration (20–100 μM) and the apparent K_m value for L-asparagine is 85 μM. In these studies, L-asparaginase activation by thiomorpholides was fully characterized by performing Lineweaver–Burk analysis and secondary replots. Hence, L-asparaginase activity was determined in the presence of different thiomorpholide compounds. All thiomorpholide compounds contain sulfur along with different substituents attached such as electron-donating, electron-withdrawing, bulky, and sterically hindered. Of the different substituents attached, the compounds having electron-donating substituents were excellent stimulators of L-asparaginase activity. None of the thiomorpholide with electron-withdrawing, bulky, and sterically hindered substituents had shown the significant activation of L-asparaginase.

The pattern of overall activation of L-asparaginase among electron-donating substituents, revealed as **4h > 4c > 4g > 4j.** The compound **4h** containing two methoxy substituents and compound **4c** having a single methoxy substituent. Hence, the resulting activation of L-asparaginase by **4h** and **4c** was higher than other compounds. This activation may be due to the electron-releasing ability of 3,4-dimethoxy substituent so that it becomes stable diamagnetic molecule. In **4h** and **4c**, both the substituents attached and thio group were crucial for L-asparaginase activation due to redox and antioxidant properties. The activation obtained in the presence of **4g** was relatively lower than **4h** and **4c**. It was due to the presence of electron-donating substituents along with bulky and sterically hindered substituents. The possible activation of L-asparaginase has been explained by using kinetic experiments and progress curve. The pattern of the lines obtained in the presence of thiomorpholides has shown direct evidence for L-asparaginase activation (Fig. 2). From the summary of kinetic parameters, it is evident that V_{max} values were increased and K_m values were decreased in the presence of thiomorpholide **4h, 4c**, and **4g (**0–100 μM**)** that corresponds to a nonessential mode of activation (Table 3). By using secondary replots ($1/\Delta$ vs 1/[thiomorpholide compound]), the values of α, β, and binding constants K_A for each thiomorpholide compound were determined (Figs. 3 and 4). The constants α and β refer to the fold change in K_m and V_{max}, respectively, in the presence of the thiomorpholides.

While, explaining the asparaginase activation by **4c** and **4h,** it is to conceive that enzyme requires an electron-releasing ability of the substituents present in the thiomorpholide compounds along with the thio group. The activation in the presence of **4h** was obtained twofold higher than that of **4c,** because of two methoxy substituents in **4h.** Hence, as electron-donating ability of thiomorpholide compound increases the fold activation of L-asparaginase also increases. Data suggest that relatively specific interaction may take place in between free thio group and enzyme as a critical determinant of these interactions as well as activation. Nevertheless, it happens only when the interacting compound also supported by electron-releasing substituents. The whole data of present work indicate that compounds **4h** and **4c** may bind to the site, which is more deficient in electrons. Through these interactions, the apparent affinity of an enzyme for substrate has been raised due to a thio group, when conformational stability attained by interaction with electron-donating substituents.

The DPPH free radical scavenging activity of the synthesized thiomorpholides is summarized (Table 4). The overall pattern of the antioxidant activity was obtained as **4g > 4h > 4c > 4f > 4a > 4d** with good to moderate antioxidant activity (Fig. 5). The SAR study revealed that the parent compound **4a** showed moderate antioxidant activity (24.32%) when compared with standard BHA (72.34%). Compounds **4g, 4h,** and **4c** substituted with electronega-

Table 3
Summary of kinetic parameters and binding constants of L-asparaginase in the presence of thiomorpholides

Kinetic parameters	Control	4h	4c	4g
α	—	1.13 ± 0.01	1.062 ± 0.02	1.030 ± 0.04
β	—	4.0 ± 0.2	2.0 ± 0.8	1.34 ± 0.02
K_A (μM)	—	0.985 ± 0.005	0.0851 ± 0.002	0.625 ± 0.003
K_m (μM)	85 ± 0.5	75 ± 0.4	80 ± 0.6	82.5 ± 0.5
V_{max} (μmol mg^{-1} min^{-1})	250 ± 5.0	1000 ± 6.0	500 ± 10	333.4 ± 0.8
V_{max}/K_m	2.94 ± 0.04	13.34 ± 0.14	6.25 ± 0.05	4.04 ± 0.4

The data shown above were an average of three different experiments

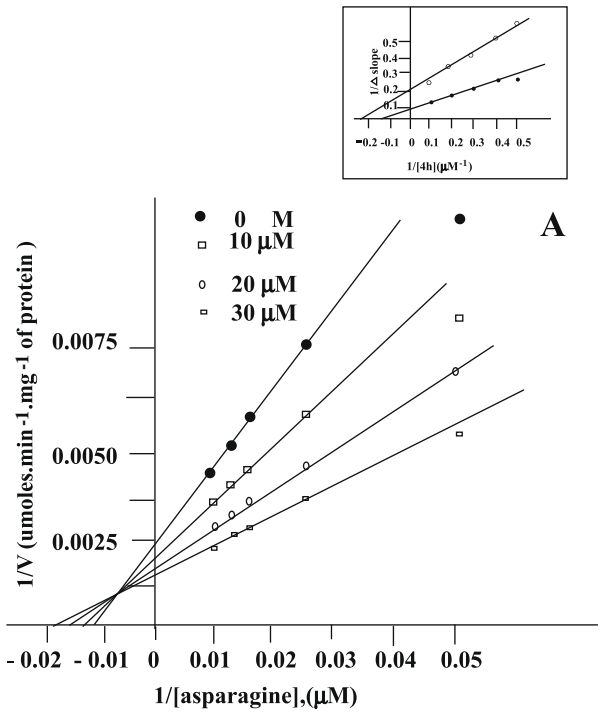


Figure 3. Lineweaver–Burk analysis of the activity shown by L-asparaginase from *Erwinia carotovora* in the presence of different concentrations of thiomorpholide **4h**. In the inset the secondary replot of ($1/\Delta$ Slope vs $1/[\mathbf{4h}]$ (μM^{-1})).

tive groups (–OCH_3 and –OH) further enhanced the activity up to 30–36% at the same concentration. The compounds **4d** and **4f** gave intermediate results (21–25%). While the remaining thiomorpholides (**4b, 4e, 4i, 4j, 4k**, and **4l**) do not show any remarkable antioxidant activity.

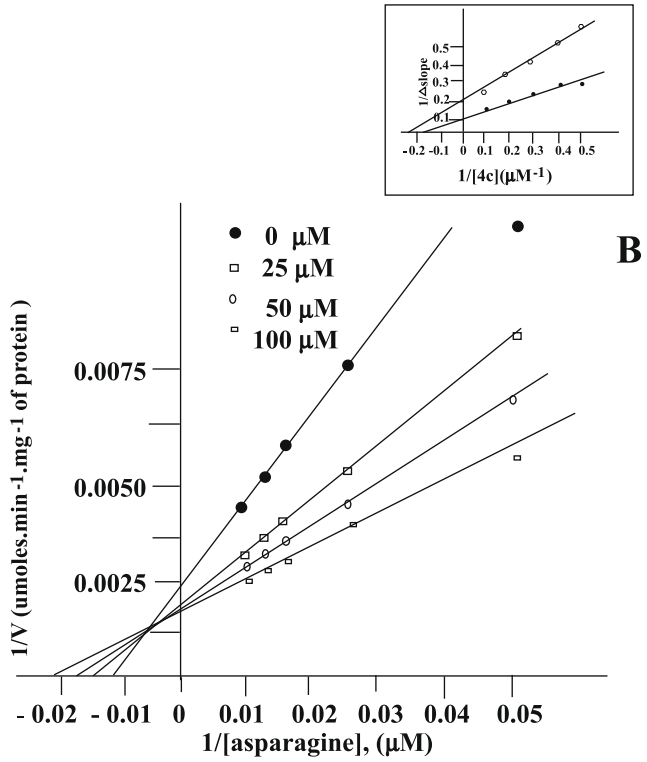


Figure 4. Lineweaver–Burk analysis of the activity shown by L-asparaginase from *Erwinia carotovora* in the presence of different concentrations of thiomorpholide, **4h**. In the inset the secondary replot of ($1/\Delta$ Slope vs $1/[\mathbf{4c}]$ (μM^{-1})).

Table 4
Percentage antioxidant activity of thiomorpholines

Compounds	% Antioxidant activity[b] (1 mM/mL)
4a	24.32 ± 0.22
4b	0
4c	24.32 ± 0.22
4d	21.59 ± 0.11
4e	0
4f	25.92 ± 0.14
4g	36.12 ± 0.17
4h	32.56 ± 0.23
4i	0
4j	0
4k	0
4l	0
BHA[a]	72.34 ± 0.51

[a] Standard substance.
[b] Mean ± SD, $n = 3$.

3. Conclusion

We report fluoroboric acid adsorbed on silica gel (HBF_4–SiO_2) catalyzed highly efficient protocol, for the synthesis of thiomorpholides by the condensation of aryl alkyl ketones with sulfur and morpholine under solvent-free conditions with excellent yields. The remarkable catalytic activity that HBF_4–SiO_2 exhibited is convincingly superior to other recently reported catalytic methods with respect to high conversions, operational simplicity, enhanced reaction rates, cleaner reaction profiles, ease of isolation of products, inexpensive and ready availability of the catalyst makes the procedure an attractive alternative to the existing methods for the synthesis of thiomorpholides. The antioxidant activity of the compounds further confirmed them as therapeutically active agents in the treatment of oxidative stress induced diseases.

Moreover, in the present study we have also introduced antioxidant thiomorpholides as activators of therapeutically important L-asparaginase. In future, these findings may also find some alternative to attain thermodynamic stability of *E. carotovora* L-asparaginase.

4. Experimental

4.1. Chemistry—general aspects

All chemicals were purchased from Sigma–Aldrich and Merck Chemical Companies. All reactions were monitored by thin layer chromatography (TLC) using aluminum plates coated with silica gel (Merck) using 10% ethyl acetate and 90% hexane as eluent. Melting points was recorded on a Buchi R-535 apparatus and are uncorrected. The products were characterized by making a comparison of their spectral and physical data with those of authentic samples. The IR spectra were recorded on Nicolet (impact 400D model) FTIR Spectrometer. The ^1H NMR spectra were recorded on a Bruker DRX-300 AVANCE at 300 MHz instrument using TMS as an internal standard and DMSO-d_6 or $CDCl_3$ as a solvent. Mass spectra were obtained by using a GC–MS Hewlett Packard (EI, 20 eV) instrument. All yields refer to isolated ones.

4.2. Preparation of catalyst

HBF_4 (1.65 g, as a 40% aqueous solution) was added to the suspension of silica gel (13.35 g, 230–400 mesh) in diethylether (40 mL). The mixture was concentrated and the residue was dried under vacuum at 100 °C for 72 h to afford HBF_4–SiO_2 (0.5 mmol g^{-1}) as a free flowing powder.[41]

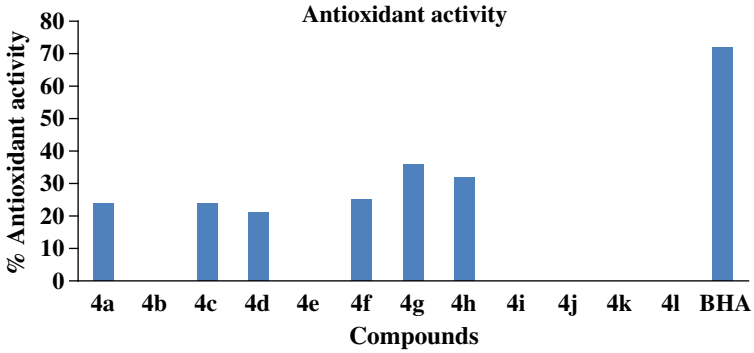


Figure 5. Graphical representation of antioxidant activity.

4.3. Experimental procedure

A mixture of acetophenone (1 mmol) with sulfur (1.2 mmol) and morpholine (1 mmol) was stirred at 80 °C in the presence of a catalytic amount of HBF_4–SiO_2 (250 mg, 5 mol %) for the appropriate time (see Table 2). After completion of the reaction as indicated by TLC, the reaction mixture was diluted with ethanol (10 ml). The filtrate and the catalyst were washed with ethanol (3 × 5 ml). The organic layer was concentrated under vacuum to afford the crude product, which on further recrystallization from ethanol afforded the pure product. The recovered catalyst was reused at least five times affording 94%, 92%, 91%, 90%, and 86% yields, respectively.

4.4. Spectral data of synthesized compounds

4.4.1. 1-Morpholino-2-phenylethanethione (4a)

Yellow solid; mp: 62–65 °C; IR (KBr): 3473, 3026, 2968, 2861, 1960, 1711, 1488, 1279, 1109, 958,707, 622 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.17–7.22 (m, 5H), 4.32 (t, 2H), 4.30 (s, 2H), 3.74 (t, 2H), 3.53 (t, 2H), 3.35 (t, 2H); MS: *m/z* 222 (M+1).

4.4.2. 2-(4-Bromophenyl)-1-morpholinoethanethione (4b)

Light yellow solid; mp: 94–95 °C; IR (KBr): 3416, 2922, 2858, 1486, 1432, 1277, 1219, 1111, 1030, 961, 722, 614 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.45 (d, 2H), 7.20 (d, 2H), 4.35 (t, 2H), 4.22 (s, 2H), 3.75 (t, 2H), 3.60 (t, 2H), 3.40 (t, 2H); MS: *m/z* 300 (M+1).

4.4.3. 2-(4-Methoxy-phenyl)-1-morpholin-4-yl-ethanethione (4c)

Oil; IR (neat): 3458, 2924, 2845, 1518, 1250, 1115, 1035, 769 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.10–7.30 (d, 2H), 6.80 (d, 2H), 4.35 (t, 2H), 4.25 (s, 2H), 3.80 (s, 3H), 3.70 (t, 2H), 3.63 (t, 2H), 3.32 (t, 2H); MS: *m/z* 252 (M+1).

4.4.4. 1-Morpholin-4-yl-2-p-tolyl-ethanethione (4d)

Colorless liquid; IR (neat): 2918, 1656, 1488, 1250, 1108, 1008, 750, 578 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.10 (d, 2H), 6.90 (d, 2H), 4.45 (s, 2H), 4.30 (t, 2H), 3.75 (t, 4H), 3.40–3.50 (t, 2H), 2.20 (s, 3H); MS: *m/z* 236 (M+1).

4.4.5. 1-Morpholin-4-yl-2-(4-nitro-phenyl)-ethanethione (4e)

Yellow oil; IR (neat): 3359, 3222, 2921, 2856, 1628, 1585, 1313, 1272, 1237, 1173 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.75 (d, 2H), 6.60 (d, 2H), 4.35 (t, 2H), 4.20 (s, 2H), 3.94 (t, 2H), 3.60 (t, 4H); MS: *m/z* 267 (M+1).

4.4.6. 2-(4-Benzyloxy-phenyl)-1-morpholin-4-yl-ethanethione (4f)

Yellow solid; mp: 107–110 °C; IR (KBr): 3465, 2923, 1649, 1583, 1510, 1431, 1118 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.30–7.40 (m, 5H), 7.0 (d, 2H), 6.86 (d, 2H), 5.03 (s, 2H), 4.28 (s, 2H), 3.88 (t, 2H), 3.67 (t, 2H), 3.55–3.63 (m, 2H), 3.32 (t, 2H); MS: *m/z* 328 (M+1).

4.4.7. 2-(2-Hydroxy-phenyl)-1-morpholin-4-yl-ethanethione (4g)

Yellow solid; mp: 75–77 °C; IR (KBr): 3689, 1627, 1489, 1211, 1117, 764 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 11.3 (s, 1H), 7.43–7.6 (m, 1H), 7.0 (d, 1H), 6.84 (d, 1H), 4.34 (s, 2H), 4.30 (t, 1H), 3.85 (t, 2H), 3.8 (t, 1H), 3.63 (m, 4H); MS: *m/z* 238 (M+1).

4.4.8. 2-(3,4-Dimethoxy-phenyl)-1-morpholin-4-yl-ethanethione (4h)

Yellow oil; IR (neat): 3525, 2923, 2850, 2751, 2590, 2288, 2045, 1650, 1593, 1482, 1340, 1270, 1112, 1025 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 6.90 (s, 1H), 6.75 (s,2H), 4.30 (t, 2H), 4.23 (s,2H), 3.85 (s,6H), 3.60–3.70 (m, 4H), 3.40 (t, 2H); MS: *m/z* 282 (M+1).

4.4.9. 1-Morpholin-4-yl-2-naphthalen-2-yl-ethanethione (4i)

Yellow solid; mp: 95–99 °C; IR (KBr): 3440, 3052, 2961, 2923, 2854, 1662, 1622, 1491, 1435, 1271, 1110 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.4–8.05 (m, 7H), 4.5 (s, 1H), 4.36 (t, 1H), 4.3 (s, 1H), 3.92 (t, 1H), 3.78 (t, 2H), 3.7 (t, 1H), 3.65 (t, 2H), 3.3 (t, 1H); MS: *m/z* 272 (M+1).

4.4.10. 2-(2-Amino-phenyl)-1-morpholin-4-yl-ethanethione (4j)

Yellow oil, IR (neat): 3368, 3240, 2820, 1618, 1532, 1400, 1328, 1262, 1208, 1029, 842, 668 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.25–7.80 (m, 4H), 4.20 (s, 2H, –NH2), 4.30–4.33 (m, 2H), 4.29 (s, 2H), 3.73 (t, 2H), 3.53 (t, 2H), 3.35 (t, 2H); MS: *m/z* 238 (M+1).

4.4.11. 2-Furan-2-yl-1-morpholin-4-yl-ethanethione (4k)

Light yellow solid; mp: 86–89 °C; IR (KBr): 3397, 2935, 2720, 2352, 1489, 1230, 1215, 1112, 774, 730 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.0–7.2 (m, 3H), 4.27 (t, 4H), 3.65 (t, 2H), 3.55 (t, 2H), 3.32 (t, 2H); MS: *m/z* 228 (M+1).

4.4.12. 1-Morpholin-4-yl-4-phenyl-butane-1-thione (4l)

Light yellow oil, IR (neat): 3473, 3112, 3011, 1642, 1490, 1210, 1115 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 7.30–7.39 (m, 3H), 7.25 (d, 2H), 6.90 (t, 1H), 6.05 (t, 1H), 3.80–3.86 (m, 4H), 3.13 (t, 4H), 2.02 (t, 2H), 1.39 (t, 2H); MS: *m/z* 283 (M+1).

4.5. Enzyme production and partial purification

The *E. carotovora* (M.T.C.C. 1428) used as a source for L-asparaginase production[42] and purified by known method.[43] All other reagents used further were of analytical grade.

4.5.1. Asparaginase activity assay

L-Asparaginase activity was determined by a modified method of Mashburn and Wriston.[44] A 0.1 ml purified enzyme solution, 0.9 ml of 0.1 M. sodium borate buffer (0.1 M, pH 8.5) and 1 ml L-asparagine (0.04 M) solution were added and incubated the same for 10 min at 37 °C. The reaction was terminated by adding

0.5 ml trichloroacetic acid (15% w/v). The reaction contents were centrifuged at 8000 rpm. The supernatant was collected and 0.2 ml of the supernatant was diluted to 8 ml with distilled water. The resulting mixture was treated with 1.0 ml of Nessler's reagent and 1.0 ml of NaOH (2.0 M). The color reaction allows to proceed for 15 min. The release of ammonia was determined by using standard curve prepared from ammonium sulfate as the ammonia source. One international unit of L-asparaginase is the amount of an enzyme that liberates 1 μmol of ammonia in 1 min at 37 °C.[45] Determinations of protein concentration were made on whole cell suspension or crude enzyme preparations by the method of Lowry et al.[46]

4.6. Kinetic measurements

In all cases, enzymatic activity was determined under defined conditions of temperature and pH. By using Lineweaver–Burk plot, the kinetic constants were determined. The activation of asparagine hydrolysis is also studied in terms of change in the values of kinetic parameters (K_m and V_{max}) in the presence and absence of thiomorpholides. In experiments employing thiomorpholides, the compound at a number of different concentrations (0, 10, 50, and 100 μM) incubated along with 0.1 ml enzyme solution, 0.9 ml sodium borate buffer (0.1 M, pH 8.5), and 1 ml L-asparagine (0.04 M) (2.5 mL final volume). The reaction was terminated with the addition of 0.5 ml trichloroacetic acid (15% w/v) and ammonia production was monitored spectrophotometrically at 37 °C for 20 min. For all thiomorpholides, identical experimental conditions were used to generate the progress curve. The Lineweaver–Burk plots and secondary replots ($1/\Delta$ Slope vs 1/[thiol compound]) were employed, to determine the values of binding constant (K_A) for respective thiomorpholides.[47,48] The Δ slope values were obtained using individual Lineweaver–Burk plots. The constants α and β refer to the fold change in the K_m and V_{max}, respectively, obtained in the presence of nonessential activator. In $1/\Delta$ Slope versus 1/[thiol] replots, the x and y intercepts correspond to $-\beta/\alpha\ K_A$ and $\beta\ V_{max}/K_m\ (\beta-\alpha)$, respectively. By assigning values for α and β, K_A values were estimated accurately.

4.7. In vitro antioxidant activity (DPPH method)

The compounds (**4a–l**) were evaluated for their in vitro free radical scavenging activity by the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method.[49] Stock solutions of different compounds (1 mM) were mixed with the DPPH methanol solution (0.5 mL, 0.3 mM) in 3 mL of total reaction mixture and allowed to react at room temperature. After 30 min. absorbance values were measured at 520 nm and converted to% antioxidant activity. For a comparative study, the Butylated hydroxyl anisole (BHA) is used as the standard. The data are summarized in Table 4.

$$\%\text{antioxidant activity} = [1 - \text{OD of test compound}/\text{OD of control compound}] \times 100$$

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