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New saccharin derivatives as tyrosinase inhibitors

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ABSTRACT

A newly series of 6-(phenylurenyl/thiourenyl) saccharin (**6a–y**) derivatives were synthesized and their inhibitory effects on the diphenolase activity of banana tyrosinase were evaluated. A 70-fold purification of the enzyme with 6.85% yield was achieved by using a Sepharose 4B-L-tyrosine-*p*-amino benzoic acid affinity column. The result showed that all the synthesized compounds inhibited the tyrosinase enzyme activity. Among the compounds synthesized, 6-(3-iodophenylthiourenyl) saccharin (**6s**) was found to be most active one (K_i = 3.95 μM) and the inhibition kinetics analyzed by Lineweaver–Burk double reciprocal plots revealed that compound **6s** was a competitive inhibitor. Structure–activity relationships study showed that generally, most of the 6-(phenylthiourenyl) saccharin derivatives (**6m–y**) exhibited higher inhibitory activity than 6-(phenylurenyl) saccharin derivatives (**6a–l**). An electron-withdrawing group at 3-position of phenylurenyl-ring increased in activity and the halogen series at 3-position of phenylthiourenyl-ring showed a qualitative relationship for higher inhibitory activity with increasing size and polarizability. We also calculated HOMO–LUMO energy levels and dipole moments of some selected the synthesized compounds (**6a**, **6h**, **6m** and **6s**) using Gaussian software.

1. Introduction

Saccharin, 1,2-benzisothiazole-3-one-1,1-dioxide, is a well-known heterocyclic compound and has been used as a sweetener in the form of its sodium salt since 1885. Yet it is also a heterocycle of pharmaceutical importance, being a key structural element of certain CNS-active drugs.[1] Saccharin compounds have been intensively investigated mainly due to its suspected cancerogenic nature.[2] Many biological activities have been attributed to this group, such as inhibitors of serine proteases,[3] cathepsin G and proteinase 3,[4] α1a and α1c adrenergic receptor antagonists,[5] human mast cell tryptase inhibitors,[6] analgesics,[7] 5-HT1a receptors,[8] aldehyde dehydrogenase inhibitors,[9] anti-anxiety and antibacterial.[10] Recently, as an orally active bioavailable human leukocyte elastase (HLE) inhibitor, [6-methoxy-4-isopropyl-3-oxo-1,2-benzisothiazole-2(3*H*)-yl]methyl-2,6-dichloro-3-[2-(4-morpholinyl)ethoxy]benzoate *S*,*S*-dioxide (WIN) series,[4] saccharin-based inhibitors, were found to have potent HLE inhibitory activity. Additionally, saccharinate complexes with amine derivatives have been useful as antidote for metal poisoning[11] and DNA-alerting ability.[12] Its importance has increased over the years and it can be viewed as a privileged scaffold in the field of medicinal chemistry. This particular heterocycle can either be a substituent of a larger compound that assumes the role of a framework, or it can play the role of the pharmacophore of bioactive molecules.[13] It was also employed in the synthesis of phenolic ether prodrugs, with the intention of increasing their oral bioavailability.[14] Being a weak acid, saccharin readily forms salts with various basic active pharmaceutical ingredients (API) thus resulting in highly soluble saccharinates, which may be used above all in pediatric medication, on account of saccharin being a potent sweetener able to mask the bitter taste of many drugs.[15] The use of saccharin acting as a co-crystal former was also reported. Behaving both like a hydrogen bond donor and acceptor, it forms highly soluble co-crystals with certain APIs.[16] In addition, certain substituted saccharin derivatives have been used as intermediates in the preparation of the 4-hydroxy-1,2-benzothiazine 1,1-dioxide (oxicam) ring system, a ring-expanded saccharin, derivatives of which possess significant anti-inflammatory properties.[17]

Since *N*,*N*′-disubstituted ureas and thioureas are linked to a series of biological activities including antiglycating,[18] MCH-R1 antagonists,[19] $P2Y_1$ receptor antagonists,[20] heparanase inhibitors,[21] anti-HIV,[22] cytostatic and antioxidant,[23] and proliferation inhibitors[24] properties, 6-aminosaccharin was reacted with isocyanates and isothiocyanates to get product unsymmetrically substituted urea and thiourea derivatives. In addition, this groups have reported[25] that aromatic sulfonamides or bis-sulfonamides possessing among others the unsubstituted urea or thiourea moieties attached to the aromatic ring, not only behave as very potent inhibitors against three isozymes, human CA I, human CA II and bovine isozyme CA, but possess an unexpectedly high affinity for the slow isozyme human CA I.

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Tyrosinase (monophenol or *o*-diphenol, oxygen oxidoreductase, EC 1.14.18.1), also known as polyphenol oxidase (PPO), is a copper-containing monooxygenase that is widely distributed in microorganisms, animals, and plants.[26] Tyrosinase could catalyze two distinct reactions involving molecular oxygen in the hydroxylation of monophenols to *o*-diphenols (monophenolase) and in the oxidation of *o*-diphenols to *o*-quinones (diphenolase).[27] Due to the high reactivity, quinines could polymerize spontaneously to form high molecular weight brown pigments (melanins) or react with amino acids and proteins to enhance brown color of the pigment produced.[28,29] Previous reports confirmed that tyrosinase not only was involved in melanising in animals, but also was one of the main causes of most fruits and vegetables quality loss during post harvest handling and processing, leading to faster degradation and shorter shelf life.[30] Recently, investigation demonstrated that various dermatological disorders, such as age spots and freckle, were caused by the accumulation of an excessive level of epidermal pigmentation.[31,32] Tyrosinase has also been linked to Parkinson's and other neurodegenerative diseases.[33] In insects, tyrosinase is uniquely associated with three different biochemical processes, including sclerotization of cuticle, defensive encapsulation and melanisation of foreign organism, and wound healing.[34] These processes provide potential targets for developing safer and effective tyrosinase inhibitors as insecticides and ultimately for insect control. Thus, the development of safe and effective tyrosinase inhibitors is of great concern in the medical, agricultural, and cosmetic industries. However, only a few such as kojic acid, arbutin, tropolone, and 1-phenyl-2-thiourea are used as therapeutic agents and cosmetic products.[32,35]

In this study series of 23 new 6-(phenylurenyl/thiourenyl) saccharin (**6a–y**) derivatives were synthesized and evaluated effect on tyrosinase, for same molecules, such as urea and thioure derivatives including aromatic sulfonamides or heterocyclic groups, have widely biological activities and pharmacologic properties.[4,8,25] Additionally, we presented structure–activity relationship analyses and molecular energy calculations of some compounds.

2. Results and discussion

2.1. Chemistry

The synthetic procedures employed to obtain the target compounds **6a–y** are depicted in Scheme 1. The 6-aminosaccharin (**5**) was synthesized from 4-nitrotoluene (**1**) in four steps by known procedures.[36] Compound **5** was reacted with phenylisocyanate or phenylisothiocyanate derivatives to get product 6-(phenylurenyl/thiourenyl) saccharin compounds (**6a–y**) in high yields.

All new compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR and MS. In the infrared spectra of compounds **6a–y**, it was possible to observe the absorptions between 3581 and 3030 cm^{-1} relating to NH stretch for urea derivatives, between 3099 and 2980 cm^{-1} relating to NH stretch for thiourea derivatives, about 3300 cm^{-1} relating to NH stretch for saccharin, absorptions in about 1725 cm^{-1} from saccharin carbonyl moiety stretch and absorptions between 1654 and 1710 cm^{-1} from urea and thiourea carbonyl moiety stretching, absorptions between 1330 and 1120 cm^{-1} relating to saccharin SO_2 stretch. The ^{1}H NMR spectra for all the synthesized urea and thiourea compounds show signals between 8.88 and 10.93 ppm relating to hydrogens attached to the nitrogen. The signals for aromatic hydrogens are between 6.62 and 8.25 ppm. In this same regions are the saccharin NH protons. Through the ^{13}C NMR data, a sign can be seen 161–162 ppm, relating to saccharin carbonyl. This is followed by the sign 152.0–153.0 ppm for urea carbonyl and 180.0–182.0 ppm for thiourea carbonyl.

2.2. Biological evaluation of synthesized compounds for tyrosinase inhibitory activity

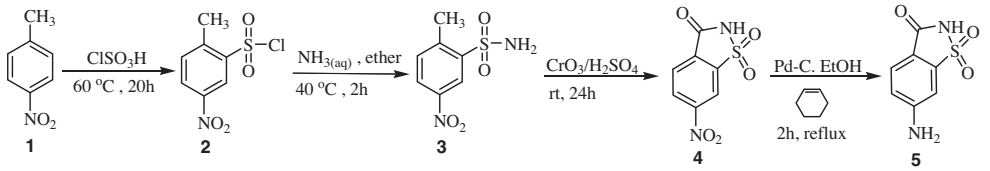
For evaluating the tyrosinase inhibitory activity, all the synthesized compounds were subjected to tyrosinase inhibition assay with catechol as substrate. The result showed that all the synthesized compounds (**6a–y**) inhibited the tyrosinase enzyme activity.

The inhibition constants of **6a–y** analogues against PPO were summarized in Table 1. We have determined the K_i values of 3.95–40.18 μM for the inhibition of banana PPO. Among the compounds synthesized, **6s** (K_i = 3.95 μM) was found to be the most active one and the inhibition kinetics analyzed by Lineweaver–Burk double reciprocal plots revealed that compound **6s** was a competitive inhibitor (Fig. 1). It was reported that DTT and sodium metabisulfite were the most effective inhibitors of banana root PPO activity, followed by L-cysteine and L-ascorbic acid. The minimum concentrations to achieve 50% inhibition were 63, 65, 322 and 437 μM respectively.[37] Acidulants, such as citric acid can inhibit PPO activity by reducing pH and/or chelating Cu in a food product.[38,39] Ascorbic acid can also be considered as an effective compound at higher concentrations. The mechanism of ascorbic acid inhibition involves the reduction of quinones generated by PPO.[40,41] It was reported that K_i value of ascorbic acid was found to be 530 μM.[42] Our results are better than this study.

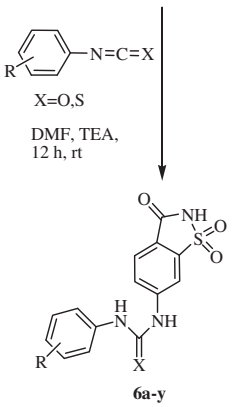
Reducing agents, antioxidants, and enzymatic inhibitors prevent browning chemically reducing the *o*-quinones. The effect of these reducing agents can be considered temporary because these compounds are oxidized irreversibly by reaction with pigment intermediates, endogenous enzymes and metals such as copper. Among sulfur containing agents, L-cystein is an effective compound to prevent enzymatic browning. Direct inhibition of polyphenol oxidase by cystein through the formation of stable complexes with copper has also been proposed.[43] Halim and Montgomery (1978) showed in a series of publications that cystein can inhibit enzymatic browning of pear juice concentrate more effectively than sulfite.[44] Kahn (1985) used cystein to inhibit browning of cut or pureed avocados and bananas.[45] As the concentration of inhibitors raised, the residual enzyme activity drastically decreased. All inhibitors manifested a similar relationship between the enzyme activity and enzyme concentration. From the progress curve obtained, all compounds (**6a–y**) showing solid lines below the lines of enzyme activity have indicative of enzyme inhibition and vice versa. The result may be related to the structure of tyrosinase contained a type-3 copper center with a coupled dinuclear copper active site in the catalytic core. The purification procedures are summarized in Table 2. As seen in Table 2, finally, PPO was purified up to 70-fold.

2.3. Structure–activity relationship (SAR)

Some notions about SAR emerged from these studies: For the synthesized urea derivatives; (a) when electron-donating groups (methoxy, methyl) at phenyl-ring compared with electron-withdrawing groups (nitro, halogen), electron-withdrawing groups at 3-position of phenyl-ring increased the tyrosinase inhibitory activity (Table 1, for inhibitory activity, **6e, 6h** and **6k** > **6b**, 6c and **6d**). The increase in electronegativities of these groups changed proportionally the effect of compounds on enzyme inhibition (for electronegativity, **NO_2** > **F** > **Cl**, for inhibitory activity, **6h** > **6k** > **6e**), (b) when the electron-withdrawing groups are at *meta*-position of compounds exhibited higher inhibitory activity than at *para*-position (compare **6e** with **6f**, **6h** with **6i**, **6k** with **6l**). For the synthesized thiourea derivatives; (c) the halogen series at 3-position of phenyl-ring showed a qualitative relationship for higher inhibitory activity with increasing size and polarizability (for size and



	6a	6b	6c	6d	6e	6f	6g	6h
X	O	O	O	O	O	O	O	O
R	H	3-OCH_3	4-OCH_3	4-CH_3	3-Cl	4-Cl	3,4-di-Cl	3-NO_2
	6i	**6j**	**6k**	**6l**	**6m**	**6n**	**6o**	**6p**
X	O	O	O	O	S	S	S	S
R	4-NO_2	2-F	3-F	4-F	H	4-CH_3	4-OCH_3	2-F
	6q	**6r**	**6s**	**6t**	**6u**	**6v**	**6y**	
X	S	S	S	S	S	S	S	
R	3-F	4-F	3-I	3-Cl	2,4-di-Cl	3,5-di-Cl	4-NO_2	



Scheme 1. Synthesis of 6-(phenylurenyl/thiourenyl)saccharin derivatives.

Table 1
Inhibitory effect of saccharin derivatives on banana tyrosinase activities

Compd	X	R	K_i (μM)	Inhibition type	Compd	X	R	K_i (μM)	Inhibition type
5	—	—	13.62	Competitive	**6l**	O	4-F	15.61	Competitive
6a	O	H	13.40	Competitive	**6m**	S	H	10.66	Competitive
6b	O	3-OCH_3	14.08	Competitive	**6n**	S	4-CH_3	9.96	Competitive
6c	O	4-OCH_3	16.55	Competitive	**6o**	S	4-OCH_3	9.81	Competitive
6d	O	4-CH_3	16.21	Competitive	**6p**	S	2-F	4.23	Competitive
6e	O	3-Cl	5.85	Competitive	**6q**	S	3-F	11.10	Competitive
6f	O	4-Cl	40.18	Competitive	**6r**	S	4-F	14.80	Competitive
6g	O	3,4-di-Cl	14.95	Competitive	**6s**	S	3-I	3.95	Competitive
6h	O	3-NO_2	16.87	Competitive	**6t**	S	3-Cl	28.30	Competitive
6i	O	4-NO_2	21.96	Competitive	**6u**	S	2,4-di-Cl	10.09	Competitive
6j	O	2-F	35.07	Competitive	**6v**	S	3,5-di-Cl	13.74	Competitive
6k	O	3-F	12.13	Competitive	**6y**	S	4-NO_2	19.53	Competitive

polarizability, I > Cl > F, for inhibitory activity, **6s** > **6t** > **6q**), (d) there was an inverse relationship between the inhibitory activity and the distance between the substituent at the phenyl ring and the sulfur atom at the thiourea group. The inhibitory activity was increased by substituent closer to sulfur atom (**6p** (R: 2-F) > **6q** (R: 3-F) > **6r** (R: 4-F) and **6u** (R: 2,4-di-Cl) > **6t** (R: 3-Cl) > **6v** (R: 3,5-di-Cl)).

2.4. Quantum chemical calculations

In order to understand the experimental results obtained, molecular calculations were performed using Gaussian software,[46a,b] some selected the synthesized compounds (**6a**, **6h**, **6m** and **6s**) (Fig. 2).

Generally, differentiation between HOMO and LUMO reflects the intensity of electron affinity, and lower differentiation suggests higher electron affinity.[47] The explaining to different effect of the synthesized urea derivatives and thiourea derivatives on tyrosinase enzyme, we performed quantum chemical calculations of **6a** and **6m** structures to calculate HOMO and LUMO energy levels and dipole moment. HOMO–LUMO energy differences ($\Delta E_{HOMO-LUMO}$) of **6m** (3.727 eV) are lower than **6a** (4.045 eV). These results compatible with inhibition effect of **6m** (K_i = 10.66 μM) and **6a** (K_i = 13.40 μM). $\Delta E_{HOMO-LUMO}$ of the compound **6h**, showed the highest inhibitory activity (K_i = 16.87 μM) among the synthesized urea derivatives, is 3.557 eV. $\Delta E_{HOMO-LUMO}$ of the compound **6s**, showed the highest inhibitory activity (K_i = 3.95 μM) among the synthesized all compounds, is 3.460 eV. Our result showed a clear relation between HOMO–LUMO energy differences and the potency of these compounds as tyrosinase inhibitors.

Additionally, the calculated dipole moment of these compounds (**6a**: 3.1473 Debye, **6h**: 4.502 Debye, **6m**: 3.8624 Debye, **6s**: 4.6391 Debye) showed a linear relationship for higher inhibitory activity with increasing dipole moment (for inhibitory activity, **6s** > **6h** > **6m** > **6a**). This relation probably to be explained that the rise in the dipole moment of the molecule increases enzyme inhibition activity for possibility of binding to copper ions in the active cite of enzyme.

3. Conclusions

In conclusion, series of new 12 urea and 11 thiourea saccharin derivatives were prepared from 6-aminosaccharin and their activities as tyrosinase inhibitors were examined. Most of phenylthio-

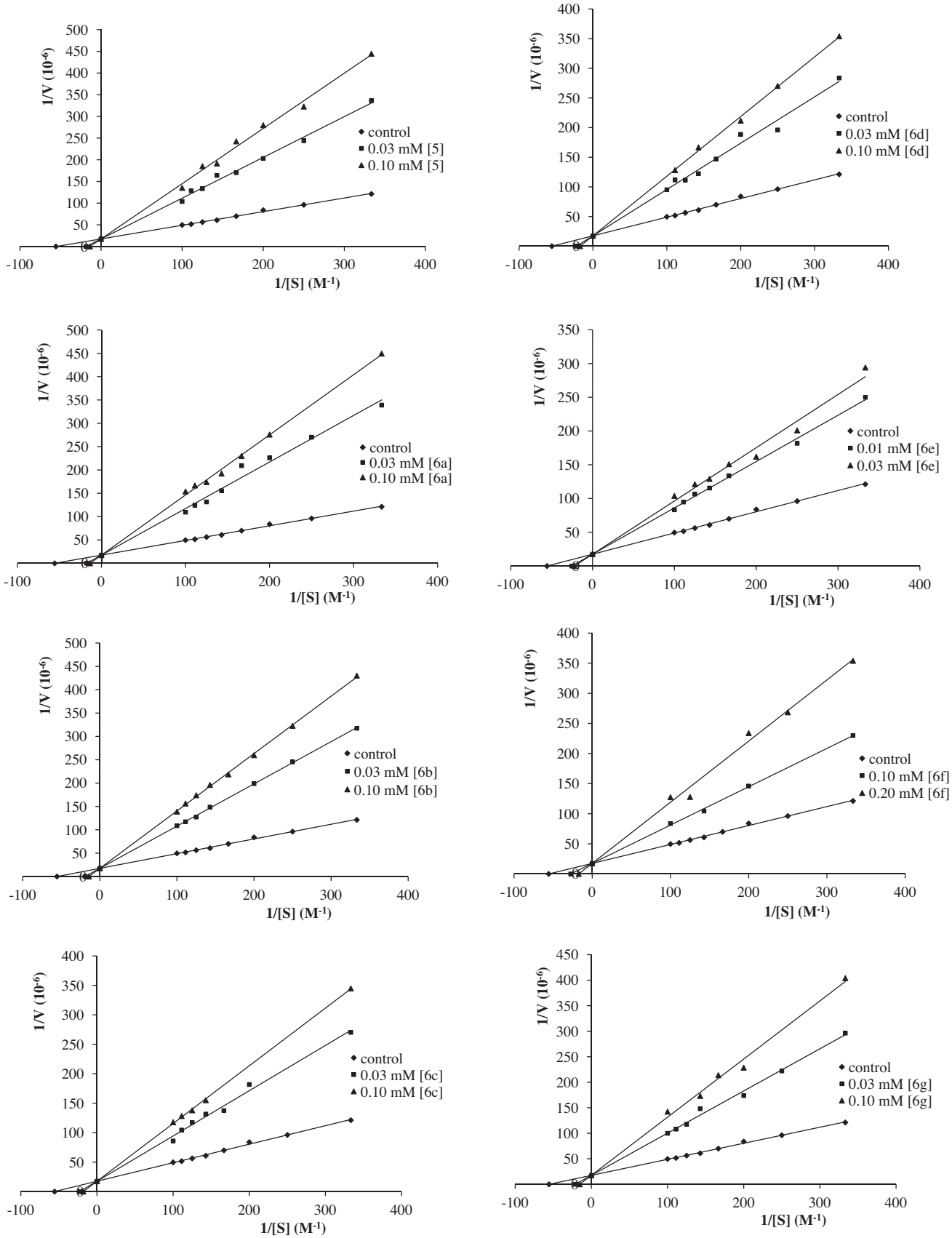
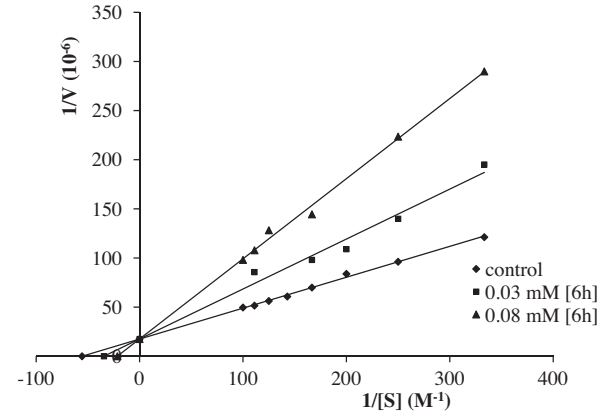
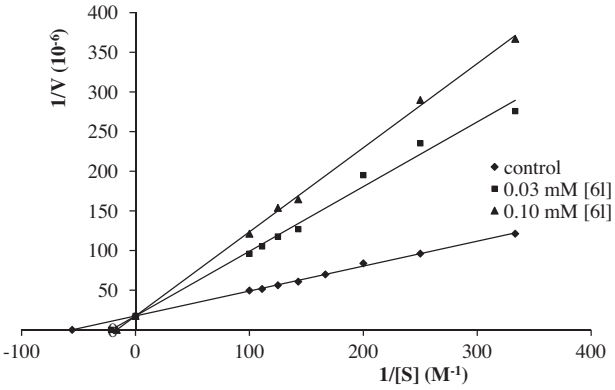
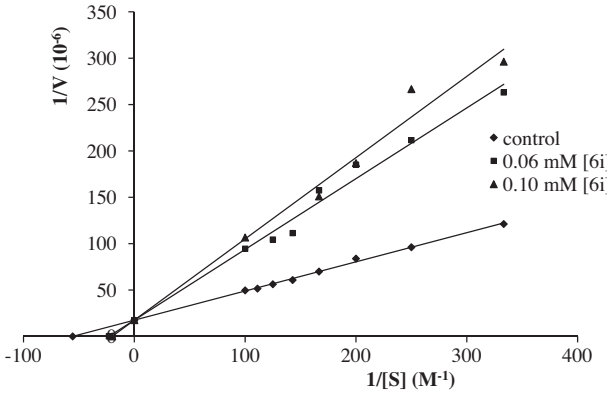
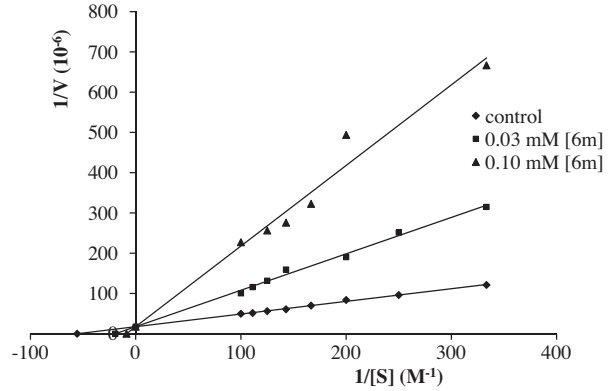


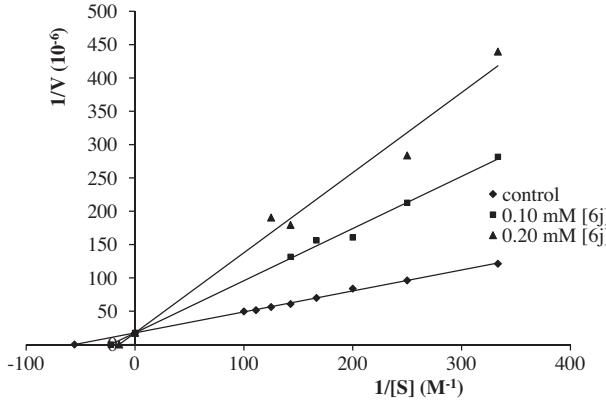
Figure 1. K_i graphics of saccharin derivatives on BPPO.

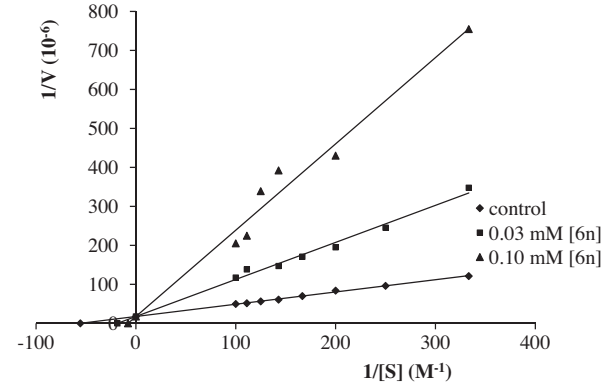


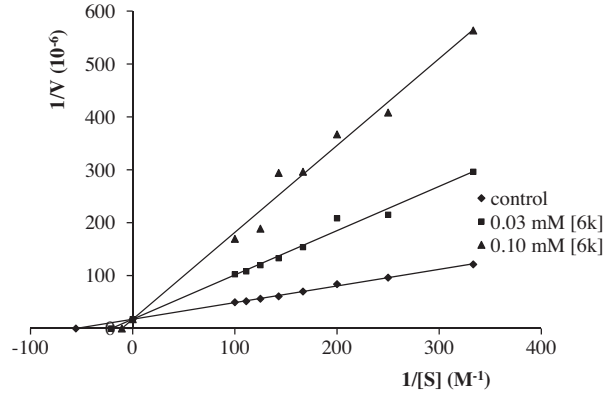












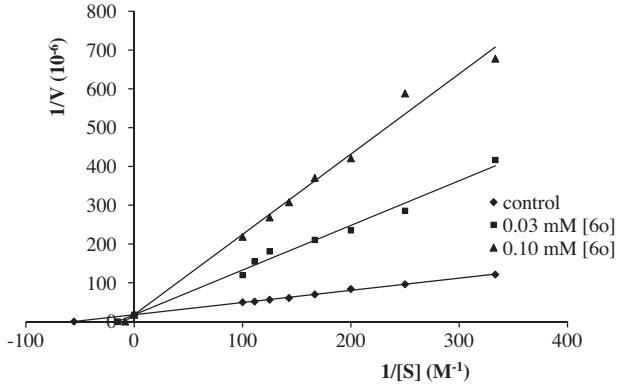


Fig. 1 (*continued*)

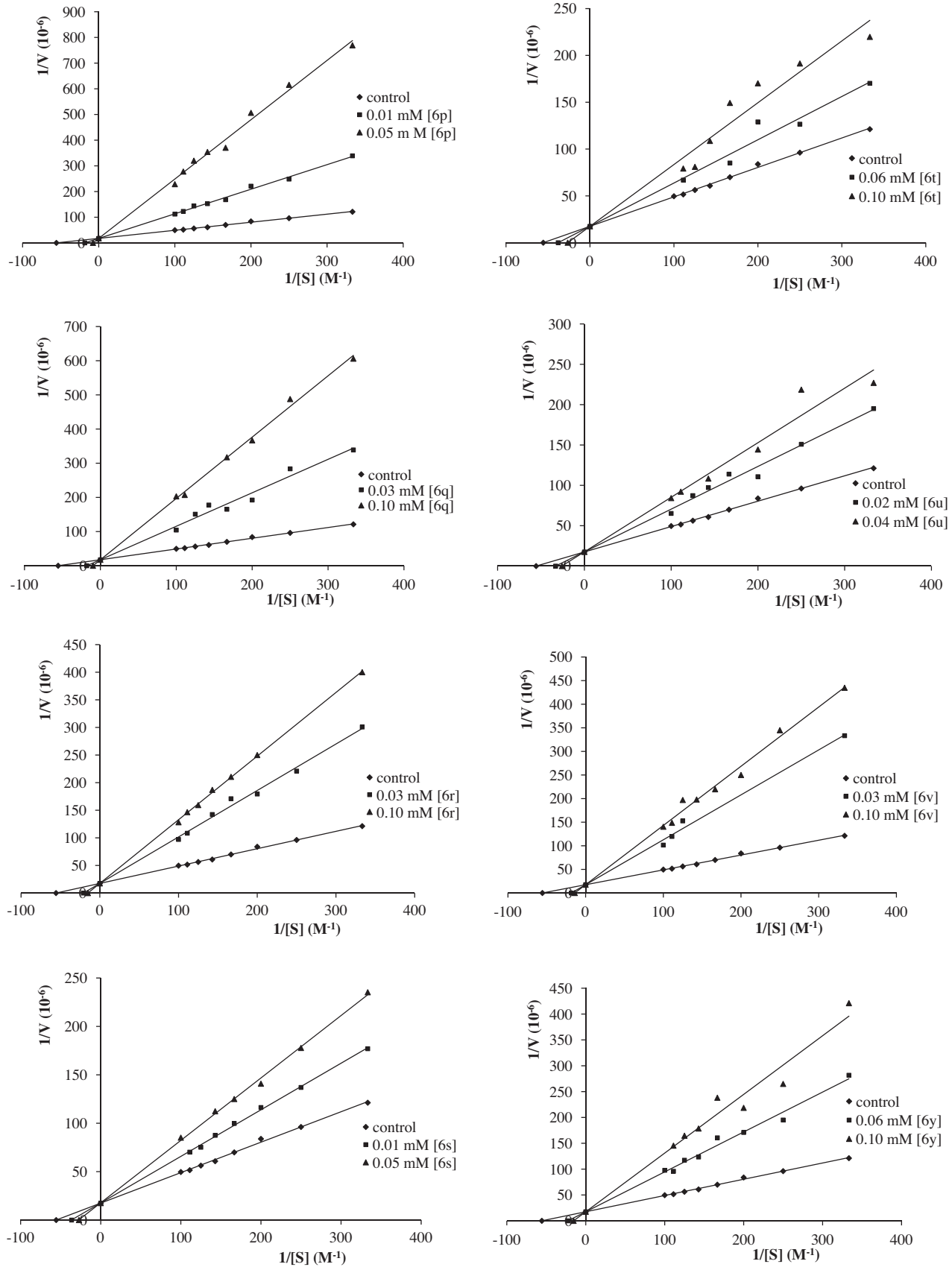


Fig. 1 (*continued*)

Table 2
Summary of the purification of tyrosinase from banana

Purification step	Volume (mL)	Total activity	Activity (U/mLdak)	Total protein (mg)	Specific activity (U/mg protein)	Overall yield (%)	Purification fold
Extract	25	502,500	20,100	0.205	24,51,219	–	–
Ammonium sulfate precipitation	10	125,250	12,525	0.045	27,83,333	24.93	1.13
Affinity chromatography	2	34,400	17,200	0.00020	1720,00,000	6.85	70.17

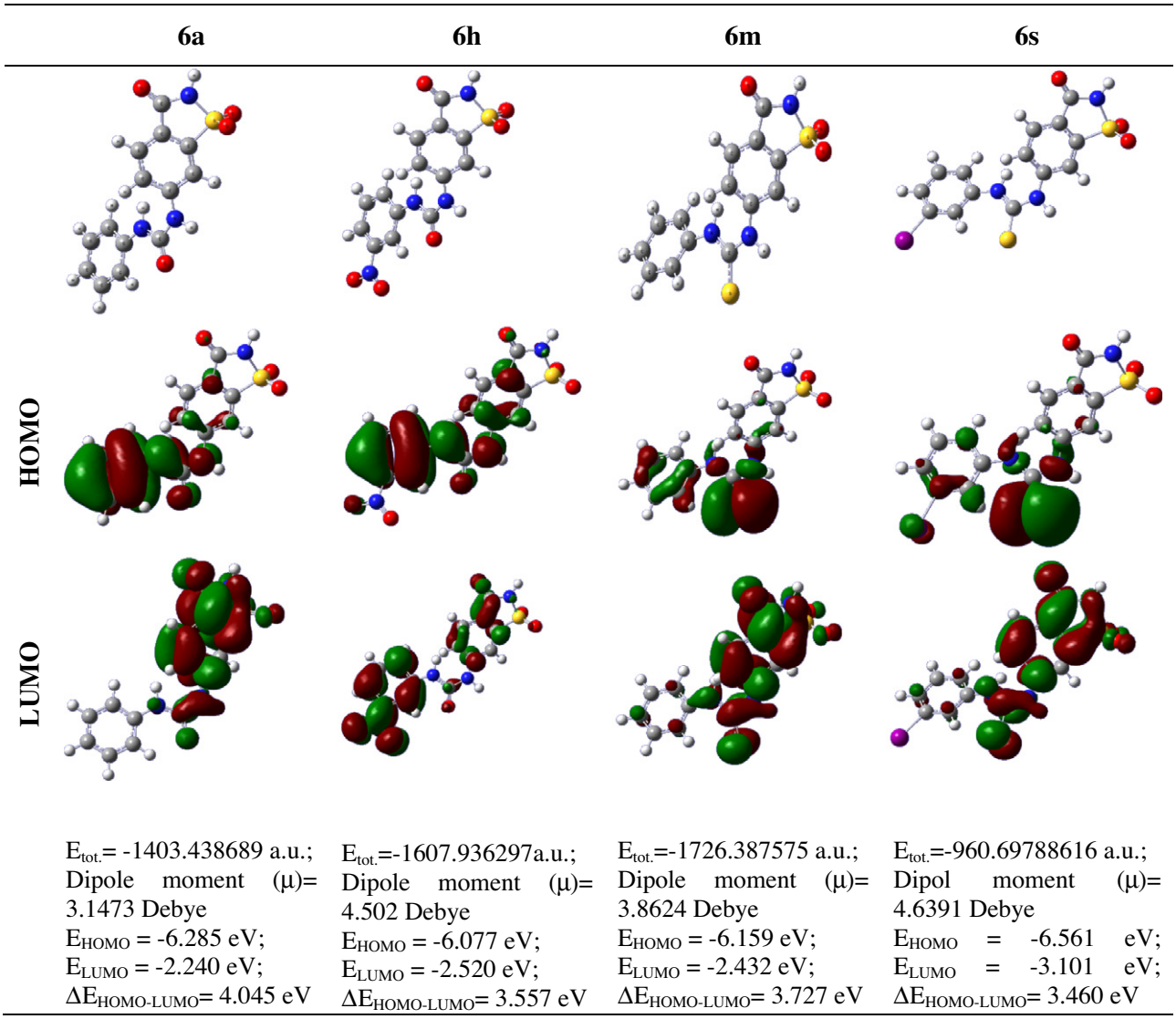


Figure 2. The calculated HOMO and LUMO for compounds **6a**, **6h**, **6m** and **6s** using HF method with 6-31G basis set.

urenylsaccharin derivatives (**6m–y**) inhibited the tyrosinase enzyme activity more than phenylurenylsaccharin derivatives (**6a–l**). The present study revealed that activity could also be influenced by the type, electronegativity and position of substituents on the phenyl ring. Among the compounds synthesized, compound **6s** showed the highest inhibitory activity. Additionally, there was a clear relation between HOMO–LUMO energy differences, dipole moment and the potency of these compounds as tyrosinase inhibitors. Kinetic study revealed that compound **6s** acted as a noncompetitive inhibitor of banana tyrosinase with K_i value of 3.95 μM.

4. Experimental

4.1. General

Melting points were taken on a Barnstead Electrothermal 9200. IR spectra were measured on a SHIMADZU Prestige-21 (200 VCE) spectrometer. ^{1}H and ^{13}C NMR spectra were measured on spectrometer at VARIAN Infinity Plus 300 and at 75 Hz, respectively. ^{1}H and ^{13}C chemical shifts are referenced to the internal deuterated solvent. Mass spectra were obtained using MICROMASS Quattro LC–MS-MS spectrometer. Solvents were dried following standard

methods. All chemicals were purchased from Merck, Alfa Easer, Sigma–Aldrich and Fluka.

4.2. Synthesis procedures and spectral data

4.2.1. 2-Methyl-5-nitrobenzenesulfonylchloride (2)

A solution of 4-nitrotoluene (13.7 g, 0.1 mol) in chlorosulfonic acid (27 mL, 0.4 mol) was stirred at 60 °C for 20 h. The solution was cooled and poured into crash ice carefully. The precipitated product was filtered and washed with abundant cold water than dried in the vacuum-oven at 40 °C. The product was provided a brown solid in 96% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm: 2.93 (3H, s), 7.69 (1H, d, J = 8.2 Hz), 8.48 (1H, d, J = 8.4 Hz), 8.92 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 20.9, 124.4, 129.4, 135.0, 143.8, 145.6, 146.3.

4.2.2. 2-Methyl-5-nitrobenzenesulfonamide (3)

To a solution of 2-methyl-5-nitrobenzenesulfonylchloride (10 g, 0.43 mol) in diethyl ether (200 mL) was added aqueous ammonia solution (35% solution, 40 mL) dropwise over 20 min keeping the temperature below 10 °C, then the mixture was stirred at 40 °C for 2 h and diethyl ether was evaporated. After the precipitated product was filtered and washed with abundant cold water, it was dried in the vacuum-oven at 40 °C. The product was provided a fawn-colored solid in 88% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm: 2.79 (3H, s), 7.22 (2H, s, NH_2), 7.52 (1H, d, J = 8.5 Hz), 8.24 (1H, d, J = 8.2 Hz), 8.80 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 20.6, 122.9, 126.0, 133.5, 143.4, 144.6, 145.7.

4.2.3. 6-Nitrosaccharin (4)

To solution chromium trioxide (20.4 g, 0.204 mol) in water (150 mL) was added concentrated sulfuric acid (190 mL) dropwise in the ice bath. 2-Methyl-5-nitrobenzenesulfonamide (10 g, 46.3 mmol) was added and the mixture was stirred at room temperature for 24 h. The mixture was filtered and the residue washed with water, dissolved in sodium bicarbonate solution and filtered under vacuum. The filtrate was acidified with concentrated HCl. After the precipitated product was filtered and washed with abundant cold water, it was dried in the vacuum-oven at 40 °C. The product was obtained a white powder in 72% yield. Mp 205.6–205.8 °C, (lit.[36] 205–207 °C); IR: 3103, 1720, 1608, 1541, 1354, 1340, 1184, 1128 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ/ppm: 8.02 (1H, d, J = 8.5 Hz), 8.56 (1H, d, J = 8.2 Hz), 8.71 (1H, s), 10.2 (1H, br, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 117.2, 126.4, 129.8, 135.2, 143.0, 151.7, 162.2.

4.2.4. 6-Aminosaccharin (5)

To a solution of 6-nitrosaccharin (1.14 g, 5 mmol) and cyclohexene (2.54 mL, 25 mmol) in ethanol (50 mL) was added 10% Pd-C (2.65 g) in the ice bath. The mixture was refluxed for 2 h with stirring, than cooled and filtered. The filtrate was evaporated to dryness. The crude product was washed with chloroform and dried under vacuum. The product was provided a yellowish solid in 87% yield. Mp 262.4–262.6 °C, (lit.[36] 264–265 °C); IR: 3473, 3377, 3244, 1708, 1598, 1285, 1163, 1107 cm^{-1}; ^{1}H NMR ($CDCl_3$+DMSO-d_6, 300 MHz) δ/ppm: 6.80 (2H, s, NH_2), 6.91 (1H, d, J = 8.5 Hz), 6.97 (1H, s), 7.60 (1H, d, J = 8.2 Hz), 9.01 (1H, br, NH); ^{13}C NMR ($CDCl_3$+DMSO-d_6, 75 MHz) δ/ppm: 103.4, 113.4, 118.4, 126.9, 142.8, 156.1, 161.8; LC–MS (*m/z*): 199.24 [MH$^+$].

4.2.5. Synthesis of 6-(phenylurenyl/thiourenyl) saccharin derivatives (6a–y)

To a solution of 6-aminosaccharin (1 mmol) and triethyl amine (1 mL) in dry DMF was added phenylisocyanate or phenylisothiocyanate derivatives (1 mmol). The mixture was stirred at room temperature for 12 h and then poured into cold 1 M HCl. The precipitate was filtered and washed with cold water. The crude products were recrystallizated from ethanol over 99% purity. Compounds **6a–y** were obtained with 69–94% yields.

4.2.6. 6-(Phenylurenyl) saccharin (6a)

White powder, 87% yield, mp 180.1–180.3 °C; IR: 3549, 3300, 3207, 1710, 1684, 1593, 1548, 1346, 1226, 1172, 1138 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.01 (1H, t, J = 7.3 Hz), 7.30 (2H, t, J = 7.8 Hz), 7.40 (1H, br, NH), 7.47 (2H, d, J = 8.3 Hz), 7.71 (1H, d, J = 8.3 Hz), 7.89 (1H, d, J = 8.5 Hz), 8.27 (1H, s), 9.02 (1H, s, NH), 9.58 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 108.9, 119.5, 120.5, 123.4, 123.5, 126.6, 129.6, 139.5, 141.7, 147.1, 152.8, 161.5; LC–MS (*m/z*): 318.09 [MH$^+$].

4.2.7. 6-(3-Methoxyphenylurenyl) saccharin (6b)

Yellowish powder, 79% yield, mp 218.2–218.4 °C; IR: 3581, 3352, 3053, 1741, 1714, 1595, 1537, 1321, 1284, 1205, 1144 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ/ppm: 3.75 (3H, s), 6.62 (1H, d, J = 7.7 Hz), 6.99 (1H, t, J = 7.9 Hz), 7.19–7.25 (3H, m), 7.73 (1H, d, J = 8.5 Hz), 7.90 (1H, d, J = 8.5 Hz), 8.28 (1H, s), 9.06 (1H, s, NH), 9.60 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 55.7, 105.2, 108.8, 108.9, 111.7, 120.7, 123.5, 126.6, 130.4, 140.8, 141.8, 147.0, 152.7, 160.4, 161.6; LC–MS (*m/z*): 348.40 [MH$^+$].

4.2.8. 6-(4-Methoxyphenylurenyl) saccharin (6c)

Yellowish powder, 76% yield, mp 251.7–252.0 °C; IR: 3500, 3344, 3030, 1731, 1708, 1597, 1527, 1348, 1255, 1207, 1134 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ/ppm: 3.73 (3H, s), 6.91 (2H, d, J = 9.0 Hz), 7.35 (1H, br, NH), 7.40 (2H, d, J = 9.1 Hz), 7.72 (1H, d, J = 8.5 Hz), 7.90 (1H, d, J = 8.5 Hz), 8.29 (1H, s), 8.88 (1H, s, NH), 9.58 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 55.8, 103.5, 108.7, 114.7, 121.4, 127.0, 132.4, 141.7, 142.7, 147.3, 152.9, 156.2, 161.6; LC–MS (*m/z*): 348.36 [MH$^+$].

4.2.9. 6-(4-Methylphenylurenyl) saccharin (6d)

Yellow solid, 82% yield, mp 240.1–240.2 °C; IR: 3523, 3331, 3041, 1758, 1709, 1595, 1537, 1338, 1226, 1132 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ/ppm: 2.23 (3H, s), 7.10 (2H, d, J = 8.2 Hz), 7.35 (2H, d, J = 8.5 Hz), 7.63 (1H, br, NH), 7.66 (1H, d, J = 8.5 Hz), 7.82 (1H, d, J = 8.5 Hz), 8.20 (1H, s), 8.90 (1H, s, NH), 9.49 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 21.1, 108.8, 119.5, 123.1, 126.2, 129.3, 129.9, 132.2, 137.0, 142.6, 146.6, 152.8, 162.6; LC–MS (*m/z*): 332.34 [MH$^+$].

4.2.10. 6-(3-Chlorophenylurenyl) saccharin (6e)

White solid, 90% yield, mp 234.6–234.8 °C; IR: 3533, 3284, 3057, 1722, 1683, 1581, 1545, 1477, 1284, 1222, 1125 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.06 (1H, dd, J = 2.1, 2.3 Hz), 7.26–7.32 (3H, m), 7.68 (1H, s), 7.72 (1H, d, J = 8.5 Hz), 7.89 (1H, d, J = 8.5 Hz), 8.24 (1H, s), 9.22 (1H, s, NH), 9.66 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 109.2, 117.9, 118.9, 120.8, 123.0, 123.8, 126.6, 131.2, 133.9, 141.1, 141.7, 146.8, 152.7, 161.5; LC–MS (*m/z*): 352.04 [MH$^+$].

4.2.11. 6-(4-Chlorophenylurenyl) saccharin (6f)

White solid, 88% yield, mp 272.7–272.8 °C; IR: 3558, 3334, 3047, 1724, 1698, 1599, 1541, 1489, 1284, 1209, 1157 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.35 (1H, br, NH), 7.38 (2H, d, J = 8.5 Hz), 7.53 (2H, d, J = 8.2 Hz), 7.76 (1H, d, J = 8.5 Hz), 7.92 (1H, d, J = 8.2 Hz), 8.28 (1H, s), 9.21 (1H, s, NH), 9.68 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 109.1, 120.6, 121.0, 123.7, 126.6, 127.0, 129.4, 138.5, 141.6, 146.9, 152.7, 161.4; LC–MS (*m/z*): 352.05 [MH$^+$].

4.2.12. 6-(3,4-Dichlorophenylurenyl)saccharin (6g)

White solid, 91% yield, mp >350 °C; IR: 3522, 3346, 3072, 1740, 1710, 1585, 1529, 1473, 1336, 1298, 1199, 1169, 1126 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.35 (1H, d, J = 9.0 Hz), 7.52 (1H, d, J = 8.8 Hz), 7.70 (1H, d, J = 8.8 Hz), 7.80 (1H, br, NH), 7.84 (1H, s), 7.87 (1H, d, J = 8.5 Hz), 8.21 (1H, s), 9.30 (1H, s, NH), 9.69 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 109.3, 119.6, 120.7, 121.1, 123.8, 124.8, 126.5, 131.3, 131.8, 139.7, 141.7, 146.5, 152.7, 161.7; LC–MS (*m/z*): 388.03 [MH^+].

4.2.13. 6-(3-Nitrophenylurenyl) saccharin (6h)

Yellow powder, 94% yield, mp 346.2–346.5 °C; IR: 3518, 3354, 3217, 1741, 1693, 1597, 1537, 1481, 1346, 1282, 1228, 1128 cm $^{-1}$; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.59 (1H, t, J = 7.9 Hz), 7.77–7.93 (5H, m), 8.27 (1H, s), 8.54 (1H, s), 9.56 (1H, s, NH), 9.75 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 109.4, 113.4, 117.8, 120.9, 124.0, 125.6, 126.6, 130.9, 140.9, 141.6, 146.7, 148.8, 152.8, 161.4; LC–MS (*m/z*): 363.13 [MH^+].

4.2.14. 6-(4-Nitrophenylurenyl) saccharin (6i)

Yellow powder, 92% yield, mp 306.4–306.7 °C; IR: 3387, 3309, 3122, 1751, 1722, 1595, 1537, 1492, 1332, 1284, 1242, 1149, 1111 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.66–7.74 (4H, m), 7.88 (1H, d, J = 8.5 Hz), 8.15 (2H, d, J = 8.7 Hz), 8.25 (1H, s), 9.75 (1H, s, NH), 9.81 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 109.4, 118.7, 120.9, 123.9, 125.7, 126.6, 141.4, 142.2, 146.2, 146.4, 152.4, 161.2; LC–MS (*m/z*): 363.11 [MH^+].

4.2.15. 6-(2-Fluorophenylurenyl) saccharin (6j)

White powder, 75% yield, mp 211.1–211.3 °C; IR: 3595, 3269, 3062, 1726, 1698, 1633, 1587, 1541, 1487, 1247, 1224, 1147 cm $^{-1}$; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.05–7.31 (4H, m), 7.70 (1H, d, J = 8.5 Hz), 7.92 (1H, d, J = 8.5 Hz), 8.11 (1H, t, J = 8.2 Hz), 8.33 (1H, s), 8.89 (1H, s, NH), 9.99 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 108.8, 115.7, 116.0, 120.7, 121.9, 123.6, 124.3, 125.3, 126.7, 127.4, 141.7, 146.8, 152.6, 161.3; LC–MS (*m/z*): 336.32 [MH^+].

4.2.16. 6-(3-Fluorophenylurenyl) saccharin (6k)

White powder, 75% yield, mp 223.2–223.5 °C; IR: 3572, 3338, 3097, 1728, 1696, 1604, 1546, 1494, 1288, 1207, 1149, 1118 cm $^{-1}$; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.13–7.20 (3H, m), 7.50 (1H, d, J = 8.2 Hz), 7.66 (1H, s), 7.73 (1H, d, J = 8.5 Hz), 7.91 (1H, d, J = 8.5 Hz), 8.27 (1H, s), 9.09 (1H, s, NH), 9.62 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 109.0, 115.9, 116.2, 117.9, 121.6, 121.8, 122.2, 123.1, 126.6, 135.7, 141.7, 147.3, 153.0, 161.3; LC–MS (*m/z*): 336.32 [MH^+].

4.2.17. 6-(4-Fluorophenylurenyl) saccharin (6l)

White powder, 75% yield, mp 237.7–237.9 °C; IR: 3572, 3338, 3097, 1728, 1696, 1604, 1546, 1494, 1288, 1207, 1149, 1118 cm $^{-1}$; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.13–7.20 (3H, m), 7.50 (2H, dd, J = 4.1, 5.0 Hz), 7.73 (1H, d, J = 8.5 Hz), 7.91 (1H, d, J = 8.5 Hz), 8.27 (1H, s), 9.09 (1H, s, NH), 9.62 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 109.0, 116.0, 116.3, 121.4, 121.5, 123.6, 126.6, 135.9, 141.9, 147.1, 152.9, 161.5; LC–MS (*m/z*): 336.32 [MH^+].

4.2.18. 6-(Phenylthiourenyl) saccharin (6m)

White powder, 81% yield, mp 171.9–172.1 °C; IR: 3356, 3091, 3049, 1735, 1705, 1533, 1494, 1323, 1240, 1170, 1136, 1124 cm $^{-1}$; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.20 (1H, t, J = 6.9 Hz), 7.32–7.45 (3H, m), 7.50 (2H, d, J = 7.3 Hz), 7.90 (1H, d, J = 8.5 Hz), 7.96 (1H, d, J = 8.5 Hz), 8.47 (1H, s), 10.47 (1H, s, NH), 10.62 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 113.5, 122.2, 124.6, 125.9, 126.0, 127.8, 129.4, 139.4, 140.6, 147.1, 161.3, 180.1; LC–MS (*m/z*): 334.30 [MH^+].

4.2.19. 6-(4-Methylphenylthiourenyl) saccharin (6n)

Yellow powder, 88% yield, mp 193.2–193.3 °C; IR: 3352, 3086, 3037, 1737, 1703, 1593, 1523, 1506, 1327, 1247, 1172, 1139, 1124 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 2.30 (3H, s), 7.19 (2H, d, J = 7.9 Hz), 7.25 (1H, br, NH), 7.37 (2H, d, J = 7.7 Hz), 7.90 (1H, d, J = 8.5 Hz), 7.94 (1H, d, J = 8.5 Hz), 8.46 (1H, s), 10.33 (1H, s, NH), 10.43 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 21.2, 113.6, 122.2, 124.8, 125.9, 127.9, 129.9, 135.3, 136.7, 140.6, 147.2, 161.4, 180.1; LC–MS (*m/z*): 348.36 [MH^+].

4.2.20. 6-(4-Methoxyphenylthiourenyl) saccharin (6o)

Yellow solid, 86% yield, mp 186.1–186.3 °C; IR: 3354, 3093, 3041, 1737, 1703, 1527, 1504, 1327, 1242, 1172, 1138, 1126 cm $^{-1}$; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 3.78 (3H, s), 6.94–7.01 (3H, m), 7.36 (2H, d, J = 8.5 Hz), 7.88 (1H, d, J = 8.5 Hz), 7.94 (1H, d, J = 8.5 Hz), 8.44 (1H, s), 10.22 (1H, s, NH), 10.35 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 55.9, 113.6, 114.5, 124.3, 125.3, 126.9, 127.6, 132.0, 142.0, 146.2, 157.6, 163.6, 180.3; LC–MS (*m/z*): 364.40 [MH^+].

4.2.21. 6-(2-Fluorophenylthiourenyl) saccharin (6p)

White powder, 69% yield, mp 157.5–157.8 °C; IR: 3365, 3080, 2930, 1718, 1696, 1597, 1531, 1490, 1294, 1259, 1232, 1163, 1138, 1116 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.21–7.33 (4H, m), 7.58 (1H, t, J = 7.9 Hz), 7.82 (1H, d, J = 8.5 Hz), 7.85 (1H, d, J = 8.5 Hz), 8.31 (1H, s), 10.07 (1H, s, NH), 10.65 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 113.6, 116.8, 123.3, 125.2, 125.8, 127.1, 127.9, 128.9, 129.4, 141.2, 146.6, 158.8, 162.0, 181.4; LC–MS (*m/z*): 352.34 [MH^+].

4.2.22. 6-(3-Fluorophenylthiourenyl) saccharin (6q)

White powder, 77% yield, mp 182.0–182.1 °C; IR: 3325, 3087, 3020, 1737, 1693, 1597, 1512, 1485, 1332, 1230, 1174, 1149, 1126 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.03 (1H, t, J = 8.2 Hz), 7.33 (1H, d, J = 8.2 Hz), 7.39–7.47 (2H, m), 7.58 (1H, d, J = 10.1 Hz), 7.94 (1H, d, J = 8.5 Hz), 8.00 (1H, d, J = 8.5 Hz), 8.51 (1H, s), 10.60 (1H, s, NH), 10.70 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 111.3, 112.5, 113.9120.1, 122.6, 126.0, 128.2, 131.0, 140.6, 141.3, 146.8, 161.4, 164.1, 180.1; LC–MS (*m/z*): 352.30 [MH^+].

4.2.23. 6-(4-Fluorophenylthiourenyl) saccharin (6r)

White powder, 90% yield, mp 202.5–202.7 °C; IR: 3331, 3097, 2980, 1718, 1694, 1597, 1539, 1496, 1330, 1288, 1165, 1143, 1105 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.23 (2H, t, J = 8.8 Hz), 7.47–7.53 (3H, m), 7.94 (1H, d, J = 8.5 Hz), 7.97 (1H, d, J = 8.5 Hz), 8.44 (1H, s), 10.35 (1H, s, NH), 10.50 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 113.8, 116.2, 122.5, 126.0, 127.3, 128.1, 135.7, 140.7, 146.9, 158.6, 161.5, 180.6; LC–MS (*m/z*): 352.34 [MH^+].

4.2.24. 6-(3-Iodophenylthiourenyl) saccharin (6s)

White powder, 86% yield, mp 175.5–175.7 °C; IR: 3313, 3092, 3045, 1736, 1695, 1577, 1506, 1467, 1330, 1232, 1172, 1145, 1124 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.18 (1H, d, J = 8.2 Hz), 7.49 (1H, d, J = 8.2 Hz), 7.55 (1H, d, J = 7.9 Hz), 7.89–7.94 (3H, m), 7.97 (1H, d, J = 8.5 Hz), 8.39 (1H, s), 10.42 (1H, s, NH), 10.59 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 94.8, 114.0, 122.5, 124.0, 126.1, 128.2, 131.3, 132.7, 134.3, 140.7, 141.0, 146.9, 161.2, 180.3; LC–MS (*m/z*): 460.56 [MH^+].

4.2.25. 6-(3-Chlorophenylthiourenyl) saccharin (6t)

White solid, 92% yield, mp 189.3–189.5 °C; IR: 3348, 3088, 3008, 1739, 1687, 1595, 1548, 1487, 1348, 1307, 1251, 1176, 1151, 1118 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.39 (1H, br, NH), 7.44 (1H, d, J = 8.5 Hz), 7.53 (1H, d, J = 8.8 Hz), 7.60 (1H, t, J = 7.6 Hz), 7.72 (1H, s), 7.92 (1H, d, J = 8.5 Hz), 7.96 (1H, d, J = 8.5 Hz), 8.42 (1H, s), 10.45 (1H, s, NH), 10.56 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 113.9, 122.5, 126.0, 126.3, 128.1, 128.4, 129.3, 129.8, 131.5, 138.4, 140.7, 146.9, 161.4, 180.3; LC–MS (*m/z*): 368.36 [MH^+].

4.2.26. 6-(2,4-Dichlorophenylthiourenyl)saccharin (6u)

White solid, 83% yield, mp 168.7–168.9 °C; IR: 3323, 3097, 3003, 1740, 1693, 1591, 1514, 1329, 1259, 1240, 1174, 1139, 1097 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.40 (1H, br, NH), 7.48 (1H, d, J = 8.5 Hz), 7.61 (1H, d, J = 8.5 Hz), 7.72 (1H, s), 7.94 (1H, d, J = 8.5 Hz), 7.99 (1H, d, J = 8.5 Hz), 8.54 (1H, s), 10.09 (1H, s, NH), 10.72 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 113.8, 122.7, 126.1, 128.3, 128.4, 129.9, 131.8, 132.4, 132.5, 135.7, 140.6, 146.7, 161.3, 181.2; LC–MS (*m/z*): 404.43 [MH^+].

4.2.27. 6-(3,5-Dichlorophenylthiourenyl) saccharin (6v)

White solid, 88% yield, mp 195.1–195.2 °C; IR: 3317, 3094, 3010, 1737, 1697, 1581, 1512, 1330, 1257, 1226, 1174, 1147, 1126 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.41 (1H, s), 7.56 (1H, br, NH), 7.62 (2H, s), 7.89 (1H, d, J = 8.5 Hz), 7.96 (1H, d, J = 8.5 Hz), 8.36 (1H, s), 10.54 (1H, s, NH), 10.73 (1H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 114.5, 122.7, 123.2, 124.9, 126.1, 128.5, 134.4, 140.8, 142.1, 146.4, 161.5, 180.5; LC–MS (*m/z*): 403.96 [MH^+].

4.2.28. 6-(4-Nitrophenylthiourenyl) saccharin (6y)

Yellow powder, 90% yield, mp 179.5–179.7 °C; IR: 3332, 3199, 3099, 1722, 1696, 1593, 1552, 1487, 1319, 1290, 1240, 1165, 1143, 1111 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ/ppm: 7.83–7.93 (4H, m), 7.97 (1H, d, J = 8.2 Hz), 8.25 (2H, d, J = 9.1 Hz), 8.39 (1H, s), 10.93 (2H, s, NH); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 114.1, 122.7, 123.0, 123.7, 125.2, 126.0, 128.2, 141.3, 143.6, 146.2, 161.9, 180.2; LC–MS (*m/z*): 379.05 [MH^+].

4.3. Tyrosinase assay

All purification steps were carried out at 25 °C. The extraction procedure was adopted from Wesche-Ebeling & Montgomery.[48] The bananas were washed with distilled water three times to prepare the crude extract, 50 g of bananas were cut quickly into thin slices and homogenized in a Waring blender for 2 min using 100 mL of 0.1 M phosphate buffer, pH 7.3 containing 5% poly(ethylene glycol) and 10 mM ascorbic acid. After filtration of the homogenate through muslin, the filtrate was centrifuged at 15,000×g for 30 min, and the supernatant was collected. A crude protein precipitate was made by adding $(NH4)_2SO_4$ to 80% saturation. The resulting precipitate was suspended in a minimum volume of 5 mM phosphate buffer and then dialyzed against 5 the same buffer overnight. The enzyme solution was then applied to the Sepharose 4B-tyrosine-*p*-amino benzoic acid affinity column,[49] pre equilibrated with 5 mM phosphate buffer, pH 5.0. The affinity gel was extensively washed with the same buffer before the banana PPO (BPPO) was eluted with 1 M NaCl, 5 mM phosphate, pH 7.0. Enzyme activity was determined; using catechol, by measuring the increase in absorbance at 420 nm according to the method Espin et al.[50] and all measurements were taken in duplicate and corrected for the non-enzymatic hydrolysis. The inhibition type of saccharin derivatives was determined by Lineweaver–Burk plots of 1/V versus 1/S at two inhibitor concentrations (Fig. 1). The inhibition constant, K_i, was deduced from the points of interception of the plots.

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