Bioorganic & Medicinal Chemistry Letters 18 (2008) 3607–3610

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

# Analogues of *N*-hydroxy-*N*′-phenylthiourea and *N*-hydroxy-*N*′-phenylurea as inhibitors of tyrosinase and melanin formation

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ARTICLE INFO

*Article history:*
Received 7 April 2008
revised 29 April 2008
Accepted 29 April 2008
Available online 4 May 2008

*Keywords:*
Melanin
Tyrosinase inhibitors
Phenylthiourea
*N*-Hydroxy-*N*′-phenylthiourea and
*N*-hydroxy-*N*′-phenylurea derivatives

ABSTRACT

A series of *N*-hydroxy-*N*′-phenylthiourea and *N*-hydroxy-*N*′-phenylurea analogues were prepared and evaluated as inhibitors of tyrosinase and melanin formation. The most active analogue **1** inhibited mushroom tyrosinase with an $IC_{50}$ of around 0.29 μM and also retained a substantial potency in cell culture by reducing pigment synthesis by 78%. Therefore, compound **1** could be considered as a promising candidate for preclinical drug development for skin hyperpigmentation application.



Mammals skin pigmentation results from the production of melanin by melanocytes and its accumulation in the epidermis. Melanin synthesis or melanogenesis is a complex pathway involving enzymatic and chemical reactions, which are restricted to melanosomes, melanocyte-specific organelles containing all components required to synthesise pigment. Among enzymes involved in melanin biosynthesis tyrosinase, a copper-containing, membrane-bound glycoprotein is the most critical and rate-limiting enzyme that catalyzes the first two steps in the biosynthetic pathway: hydroxylation of tyrosine to L-dihydroxyphenylalanine (L-DOPA) and oxidation of L-DOPA to dopaquinone.

Increased production and accumulation of melanin characterize a large number of dermatological disorders, which include acquired hyperpigmentation, such as melasma, freckles, postinflammatory melanoderma, and solar lentigo.[1,2] Many tyrosinase inhibitors find application in cosmetics and pharmaceutical products as a way of preventing overproduction of melanin in epidermis. Hydroquinone is one of the most potent whitening agents first discovered,[3,4] but since its introduction some adverse effects have been recognized. In recent years various tyrosinase inhibitors have been reported such as azelaic acid,[5] ascorbic acid derivatives,[6] arbutin,[7] kojic acid,[8] hydroxystilbene compounds like resveratrol,[9–11] and methyl ester of gentisic acid.[12] Most of the tyrosinase inhibitors are phenol/catechol derivatives, structurally similar to tyrosine or L-DOPA, which act as suicide substrates of tyrosinase.[13]

*N*-Phenylthiourea (PTU, Fig. 1) was shown to inhibit catechol oxidase enzyme that belongs with tyrosinase to the type-3 copper proteins group. The sulfur atom of the PTU binds to both copper ions in the active site of catechol oxidase and blocks enzyme activity.[14,15] Besides, our interest for chelators agents led us to study the hydroxamic acid group. Hydroxamate molecules, one of the major classes of naturally occurring metal complexing agents, have been thoroughly studied as ligands for different metal ions as Fe(III), Zn(II), and Cu(II).[16,17] The chelation involves the oxygen belonging to the carbonyl moiety and the NHOH groups. Numerous papers

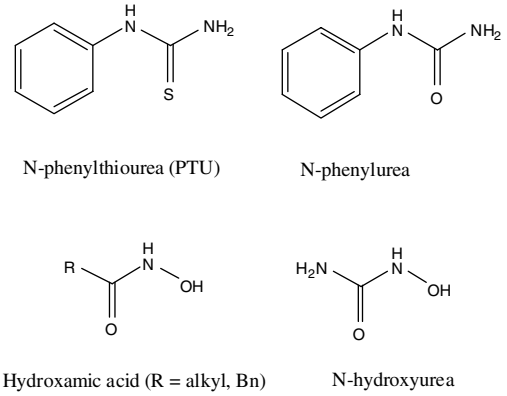


**Figure 1.** Chemical structures of *N*-phenylurea, *N*-phenylthiourea, hydroxamic acid, and hydroxyurea.

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doi:10.1016/j.bmcl.2008.04.079

showed that *N*-hydroxyurea (–NHC(O)NHOH) function seems particularly suited as hydroxamate alternative in metal-chelating ability since it incorporates the C(O)–NHOH group that is necessary to establish the same ideal type of metal chelation. *N*-Hydroxyurea itself, used as antineoplastic drug since 1960s,[18] is known to form a complex with Fe(III) and Cu(II) metal ions.[19]

In the present report, using PTU as starting point, we designed and synthesized new compounds where the primary amino group of PTU was replaced by hydroxylamino derivatives and the sulfur atom was conserved or replaced by an oxygen atom to increase $Cu^{2+}$-chelating properties of the resulting compounds, thereby generating *N*-hydroxy-*N*′-phenylthiourea and *N*-hydroxy-*N*′-phenylurea derivatives (**1**–**22**, Tables 1–3). These compounds were evaluated on tyrosinase activity in vitro and on melanin production by cultured melanocytes.

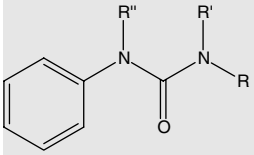
The detailed chemistry of compounds has been previously described by our group in a patent.[20] Briefly, and according to Scheme 1, a number of commercially available phenylisocyanate or phenylisothiocyanate were treated with different *N*-hydroxylamine derivatives in the presence of dimethylformamide, and triethylamine affording *N*-hydroxy-*N*′-phenylthiourea and *N*-hydroxy-*N*′-phenylurea derivatives (**1**–**14**; **18**–**22**). Compounds (**15**–**17**) were prepared according to Scheme 2, by treating phenylcarbamoyl chloride derivatives with *N*-hydroxylamine derivatives in the presence of dimethylformamide, dichoromethane and triethylamine.[21,22]

Then all these derivatives were evaluated on mushroom tyrosinase activity and their ability to inhibit melanin formation by B16 melanoma cell line was investigated. Consistent with previous reports,[23,24] PTU induced a strong inhibition of the tyrosinase activity ($IC_{50}$ = 1.8 μM) in contrast to *N*-phenylurea which showed no inhibition in our assay (Table 1). Interestingly, when the amino group and the sulfur moieties of the PTU were replaced by *N*-hydroxylamine and oxygen, respectively, the resulting compound **1** was more potent to inhibit tyrosinase activity ($IC_{50}$ = 0.29 μM) compared to PTU. Besides, tyrosinase inhibition with compound **1** was more potent than that obtained with the reference tyrosinase inhibitors, kojic acid and hydroquinone, for which the $IC_{50}$ values were, respectively, 75 and 37 μM.

Thus compound **1** was used as benchmark compound to synthesize a series of derivatives in which different substitutions were introduced at the *N*-hydroxyurea moiety (–NH–CO–NHOH) while keeping the phenyl ring unmodified (Table 1). When the terminal NHOH group was methylated on the hydroxyl (**13, 17**) or on the NH (**14, 16**) moiety, the tyrosinase activity was completely lost. Methylation on *N*′ leads to diminished tyrosinase activity, but when NHOH motif was conserved (**15**) the product had a more potent inhibition ($IC_{50}$ = 16 μM) than kojic acid and hydroquinone, but lower than compounds **1** and PTU. When the hydroxyl of the NHOH moiety was silylated with *tert*-butyldimethylsilyl group the resulting compound **22** ($IC_{50}$ = 70 μM) showed an inhibitory activity comparable to kojic acid. Acetylation of the R′ position (compound **18**) had a low inhibitory activity ($IC_{50}$ value of 170 μM) compared to **1**.

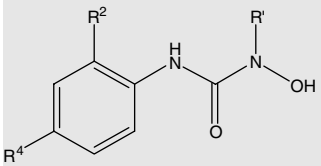
With the exception of the compound **22**, our results indicate that an unsubstituted NHOH moiety is important for inhibition of the tyrosinase activity, suggesting that the chelating ability of *N*-hydroxyurea might be important for a potent inhibition of tyros-

**Table 1**
Structures (**1, 13–18, 22**), tyrosinase, and melanin-formation inhibition

| Compound | Substituent | | | Tyrosinase inhibition | Melanin production |
|---|---|---|---|---|---|
|  | R | R′ | R″ | $IC_{50}$ (μM) | % Inhibition at 100 μM[a] |
| **1** | OH | H | H | 0.29 | 78 ± 2.12 |
| **13** | OMe | H | H | >1000 | ni |
| **14** | OH | Me | H | >1000 | ni |
| **15** | OH | H | Me | 16 | nd |
| **16** | OH | Me | Me | >1000 | nd |
| **17** | OMe | H | Me | >1000 | nd |
| **18** | OH | Ac | H | 170 | nd |
| **22** | OTBDMSi | H | H | 70 | ni |
| *N*-Phenylthiourea | | | | 1.8 | 58 ± 0.77 |
| *N*-Phenylurea | | | | >1000 | ni |
| Kojic acid | | | | 75 | 54.3 ± 1.09[b] |
| Hydroquinone | | | | 37 | — |
| Arbutin | | | | — | 43.8 ± 0.15 |

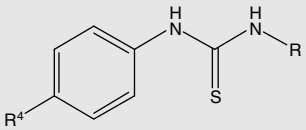
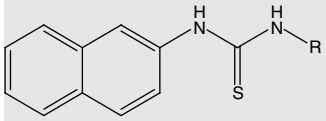
[a] Results are represented as inhibition %, means ± SE of three independent tests.
[b] Kojic acid was tested at 1 mM; ni, no inhibition; nd, not done.

**Table 2**
Structures (**1**–**12**), tyrosinase and melanin-formation inhibition

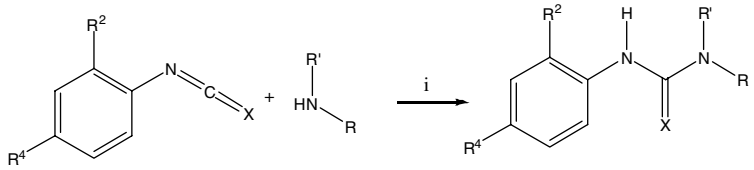
| Compound | Substituent | | | Tyrosinase inhibition | Melanin production |
|---|---|---|---|---|---|
|  | R′ | $R^4$ | $R^2$ | $IC_{50}$ (μM) | % Inhibition at 100 μM[a] |
| **1** | H | H | H | 0.29 | 78 ± 2.12 |
| **2** | H | OH | H | 41 | 19 ± 1.31 |
| **3** | H | OMe | H | 32 | ni |
| **4** | H | OMe | OMe | >1000 | ni |
| **5** | H | OBu | H | >1000 | ni |
| **6** | H | OBn | H | 6.3 | ni |
| **7** | H | $NO_2$ | H | 2.6 | 86 ± 0.44 |
| **8** | Me | $NO_2$ | H | 770 | 82 ± 0.23 |
| **9** | H | NHCONHOH | H | 27 | 66 ± 1.54 |
| **10** | H | $CF_3$ | H | 4.3 | 79.3 ± 0.58 |
| **11** | H | Br | H | 2.7 | 75.5 ± 2.30 |
| **12** | Me | Br | H | >1000 | ni |
| *N*-Phenylthiourea, *N*-phenylurea, kojic acid, hydroquinone, arbutin | | | | See Table 1 | |

[a] Results are represented as inhibition %, means ± SE of three independent tests; ni, no inhibition.

**Table 3**
Structures (**19**–**21**), tyrosinase and melanin-formation inhibition

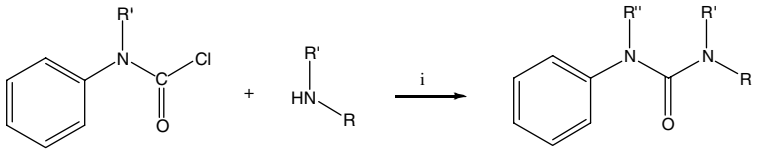
| Compound | Substituent | | | Tyrosinase inhibition | Melanin production |
|---|---|---|---|---|---|
|  | R | $R^4$ | $R^3$ | $IC_{50}$ (μM) | % Inhibition at 100 μM[a] |
| **19** | OH | H | | >1000 | ni |
| **20** | OMe | $NO_2$ | | >1000 | 75.5 ± 2.93[b] |
|  | | | | | |
| **21** | OMe | Naphtyl | | 26 | 85 ± 0.30 |
| *N*-Phenylthiourea, *N*-phenylurea, kojic acid, hydroquinone, arbutin | | | | See Table 1 | |

[a] Results are represented as inhibition %, means ± SE of three independent tests.
[b] Compound **20** was tested at 50 μM; ni, no inhibition.



X = O : **1-14, 18 22** (Tables 1, 2)
X = S : **19, 20, 21** (Table 3)
R; R' : Tables 1, 2, 3
$R^2$; $R^4$ :Tables 2, 3

**Scheme 1.** Reagents: (i) DMF, $NEt_3$.



R'' = Me : **15-17** (Table 1)

**Scheme 2.** Reagents: (i) DMF, $CH_2Cl_2$, $NEt_3$.

inase. Besides, the non-inhibitory property of *N*-phenylurea revealed the importance of the NHOH moiety for tyrosinase inhibition.

In a next set of experiments, the *N*-hydroxyurea moiety was kept unsubstituted, except in two cases for which R′ is a methyl group (**8, 12**), and modifications were done at position C-4 of the phenyl ring (Table 2). Noteworthy, a product with a –OH group at C-4 position of the phenyl ring should exhibit potent tyrosinase inhibition by acting as a competitive substrate of tyrosine.[25]

When a –OH (**2**) or –NH–CO–NHOH (**9**) group was attached to the C-4 position of the phenyl ring, the resulting compounds inhibited tyrosinase activity (respectively, $IC_{50}$ = 41 and 27 μM), but were less potent than compound **1**. Addition of a hydrophobic electrodonor –OMe moiety at C-4 position led to compound **3**. The latter showed a better inhibitory effect ($IC_{50}$ = 32 μM) compared to compound **2** that had a hydrophilic moiety at the same position. However, addition of a –OMe moiety at both C-4 and C-2 positions of the phenyl ring (**4**) prevented the ability of the compound to inhibit tyrosinase. It seems that substitution on C-2 position of the phenyl ring (**4**) diminished consequently tyrosinase activity. Similar results were observed when a –OBu group was added to the C-4 position (**5**). For the latter, the increasing length of hydrocarbon chain drastically affected tyrosinase inhibition. Addition of –OBn, $-NO_2$, $-CF_3$ or Br at C-4 position of the phenyl ring resulted in compounds **6** ($IC_{50}$ = 6.3 μM), **7** ($IC_{50}$ = 2.6 μM), **10** ($IC_{50}$ = 4.3 μM), and **11** ($IC_{50}$ = 2.7 μM), respectively, that exhibited a potent tyrosinase inhibitory activity, but lower than that of compound **1** or PTU. When R′ position was a methyl, compounds **8** and **12** lost the ability to inhibit tyrosinase activity compared to compounds **7** or **11** (R′ = H). This point is coherent with results described in Table 1, which showed that an unsubstituted NHOH m oiety was important for tyrosinase inhibition.

Noteworthy, compounds with hydrophobic electron attractor groups ($CF_3$, $NO_2$) at C-4 position showed a better efficacy than compounds with electrodonor groups (OH, OMe, OBu), except for compounds **6** and **11** (OBn, Br), which exhibited an inhibitory activity comparable to that of compounds having electroattractor group at C-4 position. Therefore, it appears that further substitu-

tions on the phenyl ring should be evaluated for a better understanding of their influence on tyrosinase activity. In conclusion, modification of the C-4 position reduced the ability of compound **1** to inhibit the activity of tyrosinase.

In the last set of experiments, compounds were prepared, replacing the oxygen of the *N*-hydroxyurea moiety by a sulfur atom affording *N*-hydroxythiourea derivatives (Table 3). Replacement of the carbonyl oxygen in compound **1** by a sulfur atom resulted in compound **19** with no inhibitory effect against tyrosinase. Additionally, methylation of the N–OH moiety and addition of a nitro group at the C-4 position of the phenyl ring generated compound **20** with no inhibitory potential. On the other hand, replacement of the phenyl ring by a naphtyl and addition of a –OMe group at R position (**21**) improved the inhibitory activity ($IC_{50}$ = 26 μM) against tyrosinase compared to compounds **19** and **20**.

The effect of the different compounds was next evaluated on melanin production. To this aim, B16 melanoma cell line was left untreated or incubated with the different compounds (Table 1–3). The results revealed that PTU strongly reduced melanin synthesis (58%). In agreement with the results on tyrosinase activity, compound **1** inhibited melanin synthesis (78%) more efficiently than kojic acid or arbutin.

Consistently, most of the compounds that lost inhibitory potential against tyrosinase did not inhibit melanin formation (**4**, **5**, **12**, **13**, **14**, and **19**) except for compounds **8** and **20**, which did not inhibit tyrosinase activity but strongly reduced melanin formation by 82% and 75.5%, respectively. We can hypothesize that either these two compounds act through a pathway different from the inhibition of tyrosinase activity to prevent melanogenesis or these differences can arise from difference between mushroom and murine tyrosinase amino acid sequence. On the other hand, compounds **2**, **3**, **6**, and **22** had an ability to inhibit tyrosinase activity stronger than kojic acid or hydroquinone but exhibited low (compound **2**) or no inhibitory effect (compounds **3**, **6**, and **22**) on melanin synthesis. This observation could be explained by their low membrane permeability properties. Finally, compounds **1**, **7**, **8**, **9**, **10**, **11**, and **21**, which were more potent tyrosinase inhibitors than reference products, were also more efficient, except for compound **9**, in inhibiting melanin production compared to kojic acid or arbutin. Noteworthy, the compounds were all assessed for their cytotoxic potential by measuring viability of B16 cells after incubation with the compounds at different concentrations. The results indicated that the compounds did not induce cytotoxic effects even at the highest concentration (100 μm) (data not shown).

It can be concluded from this whole study that NHOH moiety is important for tyrosinase inhibition. Compound **1** with an unmodified phenyl ring is the best product we have described so far. However, further studies including electroattractor group at C-4 position associated with electron-donating and/or other electro-attracting groups at other positions should be done.

Finally among the 22 compounds evaluated in this study, compound **1**, inhibiting mushroom tyrosinase with an $IC_{50}$ of around 0.29 μM and also retaining a substantial potency in cell culture by reducing pigment synthesis by 78%, may be a promising candidate for preclinical drug development for skin hyperpigmentation application.

## Acknowledgments

The authors are grateful to Dr. Martina Zsely and Dr. Corine Bertolotto for reading this manuscript.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.04.079.

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21. *N*-Hydroxy-*N*′-phenylurea (compound **1**). To a suspension of hydroxylamine hydrochloride (17.8 g, 0.248 mol) in dimethylformamide (230 mL) was added triethylamine (35 mL, 0.249 mol). The reaction mixture was cooled to 0 °C and phenyl isocyanate (25 mL, 0.225 mol) was added dropwise under $N_2$ atmosphere. The mixture was then allowed to reach room temperature and stirred for 4 h. The solvent was concentrated and the crude product was purified by column chromatography using hexane–ethyl acetate (8:2) as eluent, then recrystallized in ethyl acetate to afford **1** as a white solid (7.02 g, 20.5% yield). Mp (°C): 150–155; $^1$H NMR (250 MHz, DMSO-$d_6$): $\delta$ 8.37 (m, 3H, 2NH, –OH), 7.39–7.5 (m, 2H, Ar), 7.2–7.35 (m, 2H, Ar), 6.9 (m, 1H, Ar). MS (ESI pos): $[M+Na]^+$ $m/z$ = 175.
22. *N*′-Hydroxy-*N*-methyl-*N*-phenylurea (compound **15**). To a suspension of hydroxylamine hydrochloride (0.5 g, 6.97 mmol) in dimethylformamide (0.5 mL) and dichloromethane (7 mL) was added triethylamine (7 mmol, 0.98 mL). The reaction mixture was cooled at 0 °C and a solution of *N*-methyl-*N*-phenylcarbamoyl chloride (1.2 g, 6.97 mmol) in dichloromethane (5 mL) was added dropwise. The mixture was then allowed to reach room temperature and stirred for 2 h. The solvent was concentrated and the crude product was purified by column chromatography using hexane–ethyl acetate (1:1) to afford **15** (170 mg product, 15 % yield). Mp (°C) 105–110 $^1$H NMR (250 MHz, DMSO-$d_6$): $\delta$ 8.20 (m, 2H, NH, –OH), 7.35–7.45 (m, 2H, Ar), 6.9–7.1 (m, 3H, Ar), 3.2 (s, 3H, $-CH_3$). MS (ESI pos): $[M+H]^+$ $m/z$ = 167 and $[M+Na]^+$ $m/z$ = 189.
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