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Structure–activity relationship study of novel tissue transglutaminase inhibitors

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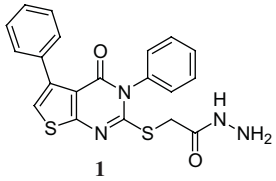
Abstract—Thieno[2,3-*d*]pyrimidin-4-one acylhydrazide derivatives were discovered as moderately potent inhibitors of TGase 2 (tissue transglutaminase) utilizing a fluorescence-based assay that measured TGase 2 catalyzed incorporation of the dansylated Lys derivative α-*N*-Boc-Lys-CH_2-CH_2-dansyl into the protein substrate *N*,*N*-dimethylated-casein. A SAR study revealed that the acylhydrazide thioether side-chain and the thiophene ring were critical to inhibitory activity.

Transglutaminases (TGases) are a family of Ca^{2+}-dependent enzymes that catalyze the formation of isopeptide bonds between the carboxamide group of protein/peptide-bound glutamine residues and the ε-amino group of protein/peptide-bound lysine residues to form N^{ε}-(γ-L-glutamyl)-L-lysine cross links with loss of ammonia. Currently, eight TGase isoforms have been identified. TGases are normally expressed at low levels in many different tissues and serve vital roles, such as in blood clotting and epithelia formation. However, it is becoming increasingly evident that some TGase isozymes are involved in diverse pathological conditions, such as celiac disease, inclusion body myositis, cataract formation, atherosclerosis and neurodegenerative disorders.[1]

The TGase 2 (i.e., tissue transglutaminase) isozyme is involved in several general biological functions, including apoptosis, cell adhesion and signal transduction.[1b] In addition, this particular isozyme has been most soundly linked to celiac disease,[2] Alzheimer's[3] and Huntington's[4] diseases. Therefore, potent and selective TGase 2 inhibitors are needed in order to further elucidate its role in various patho-physiologies and to provide lead compounds for therapeutic development.

Cystamine is a well known, albeit weak, inhibitor of TGases. Furthermore, it has shown positive effects in various cell-based and in vivo models of neurodegeneration.[1b] However, the relationship between its TGase inhibitory activity and its efficacy in cells and animals is complex and not entirely clear. All of the other reported TGase inhibitors to date do so irreversibly.[1b] In the course of screening for TGases 2 inhibitors utilizing a recently developed assay,[5] we discovered that the thieno[2,3-*d*]pyrimidin-4-one acylhydrazide derivative **1** was a moderately potent inhibitor. Herein we report an initial structure–activity relationship (SAR) study for this class of TGase 2 inhibitor.



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The 2-aminothiophene-3-carboxylates **3a** were prepared from aldehydes or ketones **2** using the Gewald reaction (Scheme 1). Depending on the starting material different reaction conditions were used (Method A:[6] R^1 = *i*-Pr or aryl, R^2 = H, Me or *i*-Pr; Method B:[7] for cyclohexanone derivatives and *N*-Boc-4-piperidone; Method C:[8] R^1 = H, R^2 = Ph). The 2-chlorothiophene **3b** was obtained from **3a** by sequential protection of the amine, chlorination of the thiophene ring with sulfuryl chloride[9] followed by amine de-protection. Reaction of

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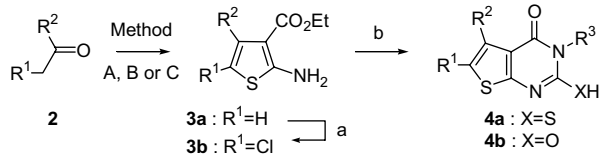


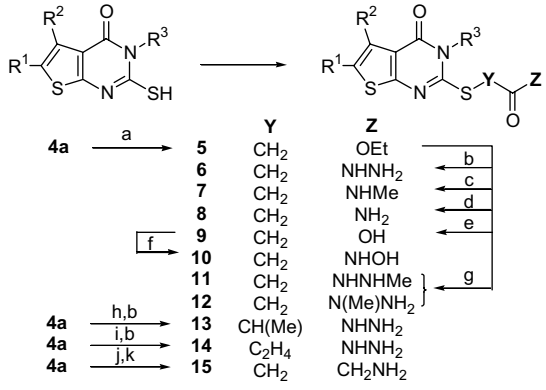
Scheme 1. Reagents and conditions: *Method A*: (i) EtO_2CCH_2CN, AcOH, $AcONH_4$, C_6H_6, Dean–Stark, Δ; (ii) sulfur, Et_2NH, EtOH, 50 °C; *Method B*: EtO_2CCH_2CN, sulfur, morpholine, EtOH, 50 °C; *Method C*: EtO_2CCH_2CN, sulfur, DBU, toluene, MW, 120 °C, 20 min; (a) TFAA, Et_3N, CH_2Cl_2 then SO_2Cl_2, CH_2Cl_2 then $NaBH_4$, EtOH; (b) R^3NCX (X = S or O), pyridine, 50 °C then MeONa, MeOH, rt.

3a–**b** with alkyl or aryl isothiocyanates (or isocyanates) in basic conditions afforded compounds **4a** (or **4b**).[10]

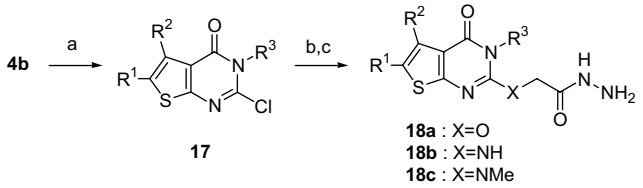
Alkylation of thiol **4a** with ethyl bromoacetate followed by aminolysis of the resulting ester **5** with hydrazine led to acylhydrazine **6** (Scheme 2). Compound **5** was also converted into amides **7** and **8**, acid **9** and hydroxamic acid **10**. Reaction of **5** with methylhydrazine led to a separable mixture of regioisomers **11** and **12**. Similarly, reaction of **4a** to methyl chloropropionate followed by aminolysis led to the acylhydrazine **13**. The homologated analog **14** was accessible by a Michael addition of **4a** with methylacrylate followed by aminolysis of the ester with hydrazine. Alternatively, alkylation of **4a** with the α-bromoketone $BrCH_2C(O)CH_2NHBoc$, **16**,[11] followed by de-protection of the amine led to the α-aminoketone **15**.

The oxygen and nitrogen analogs **18a**–**c** (Scheme 3) were obtained in three steps from **4b**. First, **4b** was converted to chloride **17** by treatment with $POCl_3$ under microwave (MW) irradiation. Addition of the requisite nucleophile to **17** followed by aminolysis of the ester gave **18a**–**c**.

Compounds **20a** and **20b**, in which the thiophene moiety has been replaced with a benzene ring, were prepared



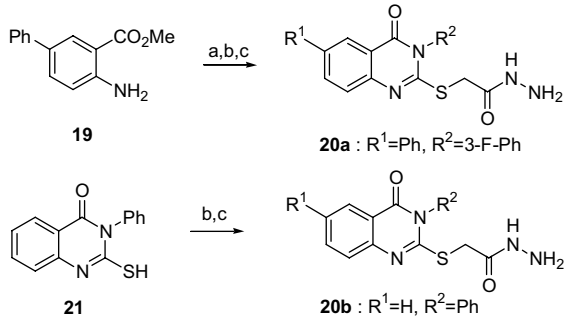
Scheme 2. Reagents and conditions: (a) $BrCH_2CO_2Et$, K_2CO_3, DMF, rt; (b) NH_2NH_2, EtOH, rt; (c) $MeNH_2$, MeOH, rt; (d) NH_3, MeOH, rt; (e) 10 N NaOH, THF/MeOH, 0 °C; (f) $(ClCO)_2$, DMF cat, CH_2Cl_2, then $NH_2OH{\cdot}HCl$, Et_3N, THF/water, rt; (g) $MeNHNH_2$, EtOH, 80 °C; (h) methyl 2-chloropropionate, K_2CO_3, THF, Δ; (i) methylacrylate, Et_3N, MeOH, 60 °C then rt; (j) **16**, K_2CO_3, DMF, rt; (k) HCl, Et_2O, rt.



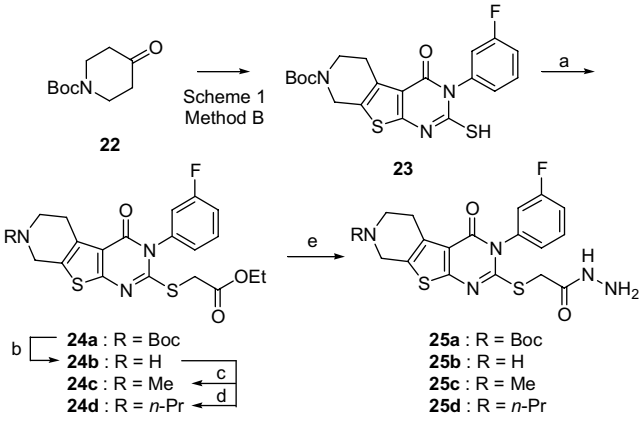
Scheme 3. Reagents and conditions: (a) $POCl_3$, MW, 150 °C, 1 h; (b) **18a**: EtO_2CCH_2OH, NaH, THF, Δ; **18b**: $MeO_2CCH_2NH_2{\cdot}HCl$, Et_3N, EtOH/MeCN, 60 °C; **18c**: $EtO_2CCH_2NHMe{\cdot}HCl$, Et_3N, EtOH/MeCN, 60 °C; (c) NH_2NH_2, EtOH, rt.

from **19** and **21** using the same procedures that were employed for the thiophene series (Scheme 4).

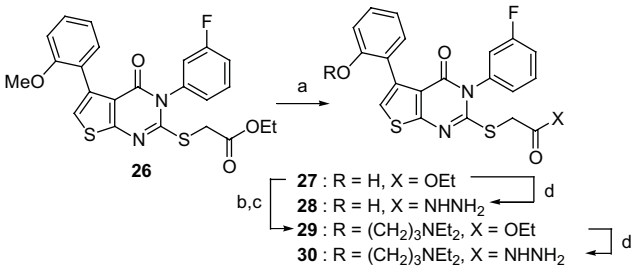
The *N*-Boc-protected thiophene derivative **23** (Scheme 5), prepared from *N*-Boc-4-piperidone, **22**, utilizing methodology described in Scheme 1/Method B, was alkylated with ethyl bromoacetate to afford **24a**. The amine was de-protected to give **24b**, which in turn was alkylated to afford **24c** and **24d**. Each of these derivatives (**24a**–**d**) was subsequently converted into acylhydrazines **25a**–**d**.



Scheme 4. Reagents and conditions: (a) 3-F-Ph-NCS, pyridine, 50 °C then MeONa, MeOH, rt; (b) $BrCH_2CO_2Et$, K_2CO_3, DMF, rt; (c) NH_2NH_2, EtOH, rt.



Scheme 5. Reagents and conditions: (a) $BrCH_2CO_2Et$, K_2CO_3, DMF, rt; (b) TFA, CH_2Cl_2, rt; (c) $H_2C{=}O$ 37%, $NaBH(OAc)_3$, AcOH, MeOH, CH_2Cl_2; (d) C_2H_5CHO, $NaBH(OAc)_3$, AcOH, MeOH, CH_2Cl_2; (e) NH_2NH_2, EtOH, rt.



Scheme 6. Reagents and conditions: (a) BBr_3, CH_2Cl_2, −78 °C to rt; (b) BrC_3H_6Cl, K_2CO_3, DMF, rt; (c) Et_2NH, KI, K_2CO_3, MeCN, 80 °C; (d) NH_2NH_2, EtOH, rt.

The phenol derivative **27** (Scheme 6) was obtained by first demethylating methyl ether **26**. Next, the hydroxyl group was utilized to introduce a diethylaminopropyl side chain in two steps to give **29**. Finally, both **27** and **29** were converted into acylhydrazines **28** and **30**.

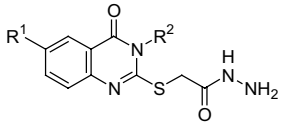
The compounds were evaluated for TGase 2 inhibitory activity utilizing a previously reported assay.[5,12] Transpeptidase activity was monitored as the increase in fluorescence intensity (FI) that accompanies incorporation of the dansylated Lys derivative α-*N*-Boc-Lys-CH_2-CH_2-dansyl (KXD) into the protein substrate *N*,*N*-dimethylated-casein (NMC). In the standard format (Std) of this assay (Tables 1–4), reactions in the presence of inhibitor were terminated after 60 min and the FI recorded. IC_{50} values were calculated using a four-parameter fit from the dependence of the FI values on inhibitor concentration.

We noted that the more potent inhibitors exhibited a phenomenon known as 'slow-binding inhibition'. The inhibitor binds to the enzyme on a time scale of minutes rather than the time scale of classical inhibitors (ms).[13] For these compounds, our standard format provides less accurate IC_{50} values. Therefore, a method was used in which full progress curves (FPC) were recorded for the TGase-catalyzed reaction of KXD with NMC in the presence of inhibitor (Tables 1–4). Reactions were initiated by enzyme addition to a solution of substrate and inhibitor. Reaction progress curves were characterized by an initial rapid velocity, frequently equal to control velocity, v_c, in the absence of inhibitor (I), followed by a first-order decrease in velocity to the final steady-state velocity, v_{ss}, that reflects the full potency of the compound. From such progress curves, IC_{50} values were calculated using the following equation: $IC_{50} = [I]/\{(v_c/v_{ss}) - 1\}$. Progress curves recorded at several inhibitor concentrations were used to determine FPC IC_{50} values (Tables 1–4). Standard errors were typically <15% of these values.

The replacement of the thiophene with a benzene ring, a known bioisoster, was briefly studied (Table 1). Compound **20b** exhibited marginal activity compared to the original inhibitor **1**. Activity was partly restored by adding a phenyl substituent at R^1 (**20a**).

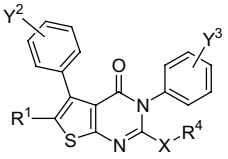
A second series of analogs was generated with the purpose of replacing the thioether and acylhydrazide

Table 1. Quinazolin-4-one derivatives prepared for structure–activity relationship studies and IC_{50} values for TGase 2 inhibition



Compd	R^1	R^2	IC_{50} (μM, Std)	IC_{50} (μM, FPC)
20b	H	Ph	18	—
20a	Ph	3-F-Ph	—	1.5

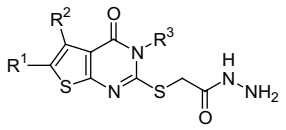
Table 2. Thieno[2,3-*d*]pyrimidin-4-one derivatives prepared for structure–activity relationship studies and IC_{50} values for TGase 2 inhibition



Compd	R^1	Y^2	Y^3	X	R^4	IC_{50} (μM, Std)	IC_{50} (μM, FPC)
1	H	H	H	S	$CH_2C(O)NHNH_2$	0.8	0.25
31	H	H	H	O	$CH_2C(O)NHNH_2$	4.5	—
32	H	H	H	NH	$CH_2C(O)NHNH_2$	—	3.7
33	Me	H	H	S	$CH_2CH_2C(O)NHNH_2$	1.3	—
34	H	H	H	S	$CH(Me)C(O)NHNH_2$	>10	—
35	H	H	H	NMe	$CH_2C(O)NHNH_2$	>20	—
36	H	4-F	4-F	S	$CH_2C(O)NMeNH_2$	>20	—
37	H	4-F	4-F	S	$CH_2C(O)NHNHMe$	>20	—
38	H	4-F	4-F	S	CH_2CO_2H	>20	—
39	H	H	4-F	S	CH_2CO_2Et	>20	—
40	H	4-F	4-F	S	$CH_2C(O)NHMe$	>20	—
41	H	2-F	3-F	S	$CH_2C(O)NH_2$	>10	—
42	H	4-F	4-F	S	$CH_2C(O)NHOH$	>20	—
43[a]	H	H	3-F	S	$CH_2C(O)CH_2NH_2$	5.3	—

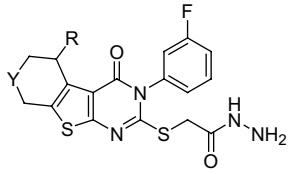
[a] HCl salt.

Table 3. Thieno[2,3-*d*]pyrimidin-4-one derivatives prepared for structure–activity relationship studies and IC_{50} values for TGase 2 inhibition



Compd	R^1	R^2	R^3	IC_{50} (μM, Std)	IC_{50} (μM, FPC)
1	H	Ph	Ph	0.8	0.25
44	H	Ph	Me	8.4	—
45	H	Ph	CH_2Ph	2.3	—
46	H	Ph	3-Py	1.2	0.48
47	H	Ph	Cy	2.6	—
48	H	Ph	2-OMe-Ph	2.0	—
49	H	Ph	3-OMe-Ph	0.82	0.47
50	H	Ph	4-OMe-Ph	2.1	—
51	H	Ph	2-Cl-Ph	1.8	—
52	H	Ph	3-Cl-Ph	1.5	—
53	H	Ph	4-Cl-Ph	1.8	—
54	H	Ph	2-F-Ph	0.50	0.16
55	H	Ph	3-F-Ph	0.25	0.18
56	H	Ph	4-F-Ph	1.3	0.32
57	H	Me	4-F-Ph	6.3	—
58	H	*i*-Pr	3-F-Ph	—	0.23
59	H	4-OMe-Ph	3-F-Ph	—	0.28
60	H	3-OMe-Ph	3-F-Ph	0.21	0.14
61	H	2-OMe-Ph	3-F-Ph	—	0.14
28	H	2-OH-Ph	3-F-Ph	1.4	—
30	H	2-(OC_3H_6-NEt_2)-Ph	3-F-Ph	4.8	—
62	H	4-F-Ph	Ph	0.80	0.29
63	H	3-F-Ph	3-F-Ph	—	0.29
64	H	2-F-Ph	3-F-Ph	—	0.14
65	Cl	2-F-Ph	3-F-Ph	—	0.13
66	Me	Ph	Ph	1.5	0.16
67	Me	2-F-Ph	3-F-Ph	—	0.15
68	*i*Pr	2-F-Ph	3-F-Ph	—	0.90
69	Ph	H	Ph	>20	—

Table 4. Thieno[2,3-*d*]pyrimidin-4-one derivatives prepared for structure–activity relationship studies and IC_{50} values for TGase 2 inhibition



Compd	Y	R	IC_{50} (μM, Std)	IC_{50} (μM, FPC)
70	CH_2	H	—	0.45
71	CH_2	Me	—	0.20
72	CH_2	Ph	0.93	0.17
73	CH_2	CH_2Ph	2.3	—
25a	*N*-Boc	H	—	1.1
25b	NH	H	—	7.1
25c	NMe	H	—	0.53
25d	*N*-*n*-Pr	H	6.0	—

moieties (X and R^4, Table 2). Replacement of the sulfur atom with oxygen (**31**) or nitrogen (**32**) resulted in a significant loss of activity. Homologation of the side chain R^4 (**33**) also led to diminished activity. All modifications of the acylhydrazide met with little success: the α- and β-methylhydrazides, acid, ester, amides and hydroxamic acid analogs (**36**–**42**) showed no inhibition at 20 μM. Only the α-aminoketone **43** exhibited some activity, albeit significantly less than the acylhydrazide.

Next, a SAR study of the R^1, R^2 and R^3 substituents was undertaken (Table 3). Replacement of the phenyl at R^3 with a methyl, cyclohexyl, benzyl or 3-pyridyl was detrimental. Methoxy or chloro substitution on the R^3 phenyl decreased activity, but introduction of a fluoro group led to equal or increased activities. Replacing the phenyl substituent at R^2 with a 2-fluorophenyl or a 2- or 3-methoxyphenyl led to a twofold increase in activity. However, an attempt to increase solubility by introducing a basic amine into the ether side-chain (**30**) resulted in decreased inhibitory activity. The R^2 phenyl group could also be replaced with an isopropyl group (**58**) with no loss in activity. Finally, substitution at R^1 was studied. Introduction of a chloro (**65**) or methyl (**66**)[14] substituent increased activity, whereas an isopropyl (**68**) was detrimental. Interestingly, transposing the phenyl group from R^2 to the R^1 position (**69**) resulted in complete loss of inhibitory activity.

Analogs bearing a fused cyclohexyl or piperidinyl moiety on the thiophene were also prepared (Table 4). Whereas the piperidinyl derivatives (**25a**–**d**) were generally less potent inhibitors, the cyclohexyl derivatives (**70**–**73**) exhibited activities more reminiscent of **1**. Introducing substituents (R = Me or Ph) on the fused cyclohexyl ring modestly increased activity.

A SAR study for TGase 2 inhibition by thieno[2,3-*d*]pyrimidin-4-one acylhydrazides revealed several interesting findings. First, the acylhydrazide thioether side-chain was crucial to inhibitory activity. Also, the thiophene ring appears to be best. The other substituents were tolerant to some changes resulting in compounds (**54**, **55**, **60**, **61**, **64**–**67**) with FPC IC_{50} values $\leqslant$0.16 μM. A subset of these compounds is currently being evaluated in TGase 2 in vitro assays utilizing different substrates (i.e., α-synuclein, β-amyloid and tau) and several cell-based assays relevant to neurodegeneration. Results of these studies and further mechanistic characterization of thieno[2,3-*d*]pyrimidin-4-one acylhydrazide TGase inhibitors will be reported in due course.

Acknowledgements

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14. Compound **66**: mp 227–228 °C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.90 (br s, 1H), 7.48 (m, 3H), 7.36 (t, 2H, J = 7.3 Hz), 7.30 (m, 3H), 7.24 (m, 2H), 3.92 (br s, 2H), 3.76 (s, 2H), 2.37 (s, 3H); ^{13}C NMR, ($CDCl_3$, 100 MHz): δ 169.2, 161.3, 157.5, 157.1, 135.2, 135.1, 134.4, 132.1, 130.4, 130.3, 130.0, 129.3, 127.9, 127.6, 119.6, 34.0, 14.1; HRESMS $[M + H]^+$: 423.0959 (calcd: 423.0944).