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Synthesis of potent water-soluble tissue transglutaminase inhibitors

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ABSTRACT

Dipeptide-based sulfonium peptidylmethylketones derived from 6-diazo-5-oxo-L-norleucine (DON) have been investigated as potential water-soluble inhibitors of extracellular transglutaminase. The lead compounds were prepared in four steps and exhibited potent activity against tissue transglutaminase.

Transglutaminases (TGases) catalyse the crosslinking of proteins by formation of an isopeptide bond between a glutamyl carboxamide in one protein and a lysyl ε-amino group of another protein. In mammals the activity is Ca^{2+}-dependent and in some family members such as TG2 (tissue transglutaminase) is inhibited by GDP/GTP binding.[1,2] Protein modification mediated by TG2 has been implicated in numerous diseases and processes[3] such as fibrosis and scarring,[4] Huntington's disease and Alzheimer's disease,[5] coeliac disease,[6] thrombosis[7] and cancer.[8] It can therefore be predicted that potent and selective TG2 inhibitors would find wide therapeutic potential. Importantly, in pathologies such as fibrosis and scarring, thrombosis and coelic disease, TG2 is likely to be acting predominantly outside, rather than inside, the cell where high Ca^{2+} concentrations lead to its activation.[9]

Several irreversible inhibitors of tissue transglutaminase (TG) have been published based upon, or related to, *N*-benzyloxycarbonyl-protected phenylalanine or *N*-benzyloxycarbonyl-protected L-glutaminylglycine, a commonly used dipeptide acyl-donor substrate.[10] These compounds generally include a spacer group terminating in an electrophilic warhead capable of covalently modifying the sulphur atom of the key cysteine residue at the active site. Examples of the warheads include maleimides,[11,12] α,β-unsaturated amides,[12–14] chloroacetamide,[12] epoxides,[13,14] thiadiazoles[15] and dihydroisoxazoles.[16] Unlike the other warheads, the dihydroisoxazole group contains a chiral centre. It was found that the 5-(*S*)-dihydroisoxazole isomers were better inhibitors of human TG2 than the corresponding 5-(*R*) stereoisomers.[17]

Sulfonium peptidylmethylketones are known to inhibit proteases irreversibly by transfer of the peptidyl portion to key active site cysteine sulphur atoms.[18] Compounds of structure Cbz–Phe–$NH(CH_2)_nCOCH_2S^+(CH_3)_2$, have been found to be potent inactivators of transglutaminases, the chain length of the $-(CH_2)_n-$ spacer moiety, $n = 3$, being optimum.[19] Here, we present a set of related inhibitors of TG2 that are derived from 6-diazo-5-oxo-L-norleucine (DON)[20] and terminate in the dimethylsulphonium methylketone moiety. The compounds to be described incorporate an extra carboxyl group compared to the previously presented sulfonium peptidylmethylketones, designed to potentially contribute to both the structure–activity profile and enhancement of their water solubility. Importantly, these inhibitors, which are permanently charged at physiological pH, have an increased likelihood of remaining outside cells, thus targeting the extracellular pool of TG2 rather than crossing the cell membrane and interacting with other intracellular transglutaminases, a factor which could be important in reducing their in vivo toxicity.[21]

The target compounds (except **4c** and **4j**) were prepared from commercially available N-protected amino acids in four steps (Scheme 1 and Table 1). The acids were converted to the corresponding *N*-hydroxysuccinimide active esters **1** by means of *N*,*N*′-disuccinimidyl carbonate in pyridine–acetonitrile.[22]

The active esters were combined with 6-diazo-5-oxo-L-norleucine ('DON' purchased from SAFC) in THF:water, 1:1 in the presence of triethylamine at 0 °C for 2 h. The solvent was evaporated at room temperature to afford the diazo intermediates **2**. These

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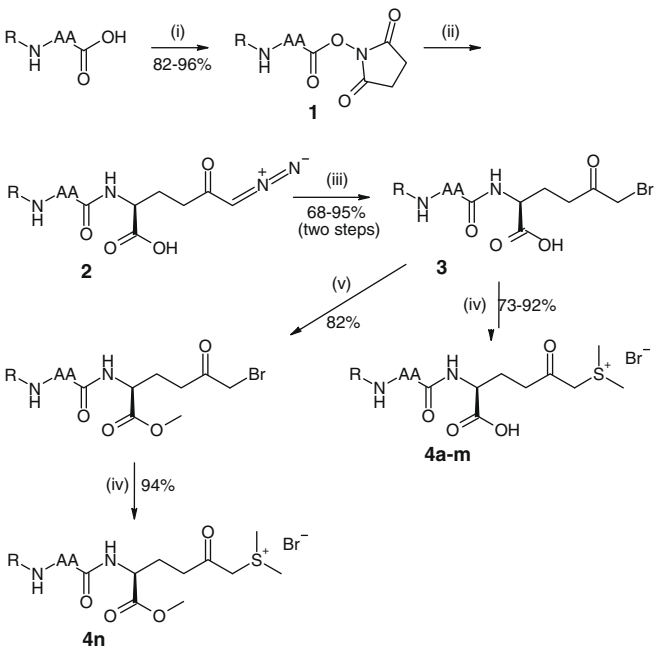

Scheme 1. Reagents and conditions: (i) *N,N′*-disuccinimidyl carbonate/pyridine/acetonitrile; (ii) DON/THF/water/Et_3N/0 °C; (iii) HBr/AcOH/EtOAc; (iv) Me_2S/MeOH; (v) TMS–CH_2N_2/THF/MeOH/0 °C.

were redissolved immediately in ethyl acetate and treated with HBr:acetic acid 1:1. Work-up gave the bromomethyl ketones **3**. These were dissolved in dry methanol and treated with dimethyl sulphide (5 equiv) for 2 days at room temperature. After removal of the solvent the residue was dissolved in water, washed with ethyl acetate and the water layer was freeze-dried to give the target compounds **4** as white powders.[23] The N-protected phenylalanine building blocks required for compounds **4c** and **4j** were prepared as follows: 2-Phenyl ethanol was converted into the corresponding 4-nitrophenyl carbonate[17] (79%). This was reacted with phenylalanine to give the stretched Cbz-protected phenylalanine[24] (89%) that was taken through to **4c**. Phenylalanine was reacted with the *N*-hydroxysuccinimide active ester of phenyl-acetic acid to give the shortened version of Cbz–phenylalanine (92%). This was carried forward to compound **4j**. Using Cbz–phenylalanine as a scaffold, compounds **5**–**7** were prepared to vary the nature of the electrophilic warhead (Fig. 1). Compound **5** was prepared in the same manner as described above but replacing dimethyl sulphide by diethyl sulphide in the final step (85%). 1,3,4,5-Tetramethylimidazole-2(3*H*)-thione[25] was alkylated with the bromomethyl ketone **3** (R = Cbz and AA = phenylalanine) to give the imidazolium compound **6** (88%). Compound **7** was prepared following published procedures.[17] The known TG2 inhibitor **8**[26] was included for reference. In addition, to investigate the role of the carboxyl group in compounds of type **4**, a methyl ester version was prepared in two steps from the Cbz phenylalaninyl bromomethyl ketone **3**: treatment with trimethylsilyldiazomethane[27] in THF–methanol at 0 °C for 3 h gave the corresponding methyl ester; the subsequent action of dimethyl sulphide in dry methanol gave the target compound **4n**.

Table 1
Inhibition data for compounds prepared as TG2 inhibitors

Compound	R	AA	IC_{50}[a] (μM)	Aqueous solubility (mg/mL)
4a	Cbz	Proline	5 ± 1	500
4b	Cbz	Glycine	8 ± 1	1000
4c	$PhCH_2CH_2OC(=O)$	Phenylalanine	8 ± 1	
4d	Cbz	Phenylalanine	10 ± 2	167
4e	Cbz	Tryptophan	12 ± 2	
4f	Cbz	(2*S*,4*R*)-4-hydroxy-2-pyrrolidine carboxylic acid	18 ± 3	
4g	Cbz	Alanine	20 ± 3	
4h	Cbz	Aspartic acid	28 ± 5	
4i	Fmoc	Phenylalanine	30 ± 4	
4j	$PhCH_2C(=O)$	Phenylalanine	110 ± 15	
4k	Cbz	Lysine	150 ± 25	
4l	*t*-BOC	Phenylalanine	300 ± 40	
4m	Cbz	Isoleucine	350 ± 42	
4n	Cbz	Phenylalanine	5 ± 1	67
5		See Figure 1	20 ± 2	
6		See Figure 1	3 ± 1	
7		See Figure 1	>200	<1
8		See Figure 1	4 ± 1	200

[a] IC_{50} values are mean values ± standard errors from a minimum of three experiments.

The compounds were characterised by melting point, infra-red spectroscopy, mass spectrometry (+ve electrospray accurate mass), proton NMR spectroscopy and carbon-13 NMR spectroscopy and had properties consistent with the proposed structures.

TG2 activity was measured using an enzyme linked sorbent assay to measure the covalent incorporation of biotinyl-5-pentylamine into *N,N′*-dimethylcasein as described previously.[28] Briefly, a 96-well microtitre plate was coated with *N,N′*-dimethylcasein and a reaction mix containing TG2 (2 ng ml^{-1}), biotinyl-5-pentylamine (132 μM), $CaCl_2$ (5 mM) and DTT (5 mM) with varying concentrations of inhibitor, was added to each well and incubated for 1 h at 37 °C. The amount of incorporated biotinyl-5-pentylamine was quantitated by reaction with Extravidin® peroxidase and colour development with *o*-phenylenediamine. The IC_{50} was expressed as the inhibitor concentration at which 50% inhibition of TG2 activity was observed. The inhibition data against guinea pig liver TG2 for compounds **4**–**8** are summarised in Table 1. Parallel studies were also undertaken using recombinant human TG2 (rhTG), using compounds **4a** and **4d**, which gave IC_{50}s comparable to those of guinea pig TG2. This was not unexpected, given the very highly conserved structures of their active sites.

To confirm the irreversible modification of TG2 with this series of compounds, compound **4a** was pre-incubated with TG2 in the presence of Ca^{2+} for different time periods up to 60 min at 20 °C. It was then diluted 100-fold and residual TG2 activity determined using the above assay. At the IC_{50} concentration of 5 μM, compound **4a** reduced activity by 77 ± 7% after 60 min preincubation (data not shown). It was noted that Ca^{2+} was required for reaction between TG2 and inhibitors, consistent with the requirement of this cofactor for the transglutaminase activity of TG2.

Compounds **4** were indeed found to be water-soluble (Table 1; data presented for **4a**, **4b**, **4d** and **4n**), very much more so than compound **7** and even comparable to or exceeding that of the reference compound **8**. As expected, the methyl ester **4n** was somewhat less soluble compared to its parent compound **4d**.

Using the phenylalanine compound **4d** as a reference point it can be seen that increasing the size of the dimethyl sulphonium group to the diethyl analogue **5** halved the activity, presumably through steric effects. In contrast, employing the tetramethylimidazolium group (analogue **6**) increased the activity to give the most potent compound in this study. Given that this moiety is at least as large as the diethyl sulphonium group it may be that a difference in chemical reactivity between the two electrophilic warheads reflects the difference in observed activity. Compound **7** contains the dihydroisoxazole pioneered by Khosla's group.[16] It has the electrophilic group on a shorter tether compared with

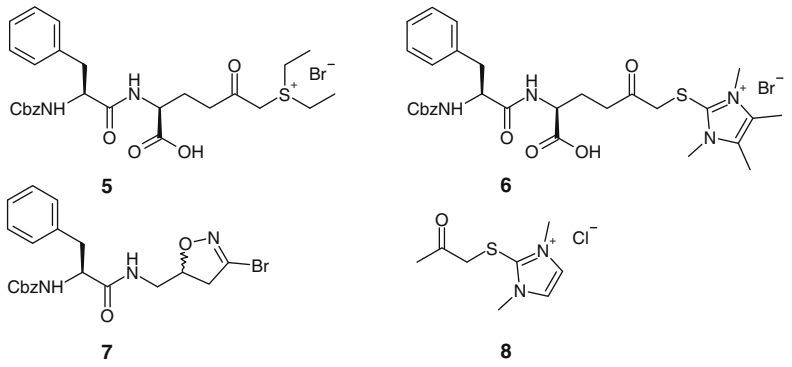

Figure 1. Variation in the electrophilic warhead.

4d, **5** and **6** and so it is not a direct comparison of the warheads' efficacy. Nevertheless, in our assay it proved to be relatively inactive.

From the compounds derived from phenylalanine it is clear that the optimum derivatisation on the N-terminus is a carbamate of the Cbz kind. This is in agreement with the work of Chica et al.[1] who demonstrated that Cbz-Gln-Gly, but not BOC-Gln-Gly is an acyl donor substrate for guinea pig liver Tgase. Moving to Fmoc (**4i**), *t*-BOC (**4l**) or shortening the Cbz to the amide version (**4j**) increased the IC_{50}. Lengthening the Cbz group by one methylene unit (**4c**) gave a slight improvement in the IC_{50} value over that for the Cbz compound **4d**. Swapping the phenyl group of **4d** for the larger indole ring **4e** made little change to the activity. Removing the phenyl ring entirely from **4d** to give the alanine analogue **4g** halved the activity and extension of the alkyl side chain to that of the isoleucine derivative **4m** effectively abolished the activity. In contrast, removal of the whole sidechain to give the glycine analogue **4b** made no change to the activity. Blocking the carboxyl group of **4d** as the methyl ester not only reduced its water solubility but also led to a small increase in activity, suggesting that the ionised carboxyl group is not playing a role in the efficacy of the inhibitors. Restraint of the flexibility of the inhibitor in the form of the proline analogue **4a** gave a small increase in activity compared to the glycine and phenylalanine compounds (**4b** and **4d**). Addition of a hydroxyl group (the hydroxyproline analogue **4f**) reduced the activity.

These results are consistent with the compounds in this study adopting two different binding modes to the enzyme. It is proposed that the Cbz-related protected analogues having aromatic sidechains (**4c**, **4d** and **4e**) interact with a hydrophobic pocket suitable for flat aromatic ligands. The loss of the aryl group (**4g**) relinquishes this stabilisation. Its conversion to a bulky aliphatic group (**4m**) destabilises the complex. The proline compound **4a** is constrained to a more angular overall shape that would naturally be adopted by the other compounds and it is possible that this drives a different binding mode in this case. The glycine analogue **4b** is one of the most active compounds in this study but the complex with the enzyme cannot attain the hydrophobic stabilisation proposed for **4c**, **4d** and **4e**. It is, however, the most flexible of all the compounds and may therefore be able to engage in the binding mode preferred by the proline compound.

The use of reference compound **4d** has so far shown no signs of cell toxicity in many of the studies it has been cited. Incubation of the compound with endothelial plasma membrane monolayers indicated no penetration of the compound over a 5-h period.[21] Their lack of cellular penetration may be a contributing factor to their lack of toxicity in that only the extracellular transglutaminases are targeted.

In a recent study to test the efficacy of **4d** in an animal model of renal scarring, a 90% reduction in scarring and significant protection of kidney function was observed. Compound **4d** demonstrated no obvious signs of toxicity.[29] Given the increasing importance of TG2 as a medicinal target and the work demonstrated here and by others, it is not unreasonable to suggest these studies may eventually lead to an effective clinical treatment of those disorders where TG2 has a role.

Spectroscopic data for *N*-benzyloxycarbonyl-L-prolinyl-6-dimethylsulphonium-5-oxo-L-norleucine bromide (**4a**)

^{1}H NMR (250 MHz, DMSO-d_6, δ DMSO = 2.50 ppm): 1.69–1.89 (m, 4H, –NCH$_2$C*H*$_2$CH$_2$– and NCH$_2$CH$_2$C*H*$_2$–), 2.01–2.23 (m, 2H, –CHC*H*$_2$CH$_2$–), 2.61–2.83 (m, 2H, –CHCH$_2$C*H*$_2$–), 2.91 (s, 6H, –S$^+$(C*H*$_3$)$_2$), 3.32–3.52 (m, 2H, –NC*H*$_2$CH$_2$CH$_2$–), 4.13-4.33 (m, 2H, –N(CH$_2$)$_3$C*H*– and –C*H*CH$_2$CH$_2$–), 4.70–4.92 (m, 2H, –C(=O)C*H*$_2$S$^+$(CH$_3$)$_2$), 5.01–5.16 (m, 2H, PhC*H*$_2$O–), 7.29–7.43 (m, 5H, Ph), 8.36 (d, *J 7.8* Hz, 1H, –N*H*–), 12.80 (br s, 1H, –COO*H*) ppm. ^{13}C NMR (63 MHz, DMSO-d_6, δ DMSO = 39.5 ppm): 23.1, 23.9 and 24.4 ((–S$^+$(*C*H$_3$)$_2$, –NCH$_2$*C*H$_2$CH$_2$– and –NCH$_2$CH$_2$*C*H$_2$–), 30.0, 31.1 and 37.7 (–N*C*H$_2$CH$_2$CH$_2$–, –*C*HCH$_2$CH$_2$– and –CHCH$_2$*C*H$_2$–), 46.6, 50.5 and 53.6 ((–C(=O)*C*H$_2$S$^+$(CH$_3$)$_2$, –*C*HCH$_2$CH$_2$– and N(CH$_2$)$_3$*C*H–), 65.9 (PhCH$_2$O–), 127.1, 127.7 and 128.5 (Ar *C*H), 137.0 (Ar *C*), 154.1 (PhCH$_2$O*C*(=O)–), 172.2 and 173.1 (*C*OOH and –*C*(=O)NHCHCOOH–), 201.6 (–*C*(=O)CH$_2$S$^+$(CH$_3$)$_2$). High-resolution MS (+electrospray): *m*/*z*: found 437.1747; calcd for $C_{21}H_{29}N_2O_6S$, 437.1746.

Spectroscopic data for *N*-benzyloxycarbonyl-L-phenylalanyl-6-dimethylsulphonium-5-oxo-L-norleucine bromide (**4d**)

^{1}H NMR (250 MHz, DMSO-d_6, δ DMSO = 2.50 ppm): 1.77–1.93 (m, 1H, –CHC*H*$_2$CH$_2$–), 2.01–2.17 (m, 1H, –CHC*H*$_2$CH$_2$–), 2.65–2.81 (m, 3H, –CHCH$_2$C*H*$_2$– and PhC*H*$_2$CH–), 2.90 (s, 6H, –S$^+$(C*H*$_3$)$_2$), 3.00–3.09 (m, 1H, PhC*H*$_2$CH–), 4.21–4.35 (m, 2H, PhCH$_2$C*H*– and –C*H*CH$_2$CH$_2$–), 4.70–4.85 (m, 2H, –C(=O)C*H*$_2$S$^+$(CH$_3$)$_2$), 4.88–5.01 (m, 2H, PhC*H*$_2$O–), 7.15–7.40 (m, 10H, Ph), 7.57 (d, *J* 8.5 Hz, 1H, ZN*H*–), 8.45 (d, *J 8.3* Hz, 1H, –C(=O)N*H*CHCOOH–), 12.90 (br s, 1H, –COO*H*) ppm. ^{13}C NMR (63 MHz, DMSO-d_6, δ DMSO = 39.5 ppm): 24.5 (–S$^+$(*C*H$_3$)$_2$), 37.3, 37.6 and 40.4 (–CHCH$_2$CH$_2$–, PhCH$_2$CH– and –CHCH$_2$CH$_2$–), 50.9, 53.6 and 56.1 (–C(=O)CH$_2$S$^+$(CH$_3$)$_2$, –CHCH$_2$CH$_2$– and PhCH$_2$CH–), 65.3 (PhCH$_2$O–), 126.3, 127.5, 127.8, 128.1, 128.4 and 129.3 (Ar *C*H), 137.0 and 138.1 (Ar *C*), 156.0 (PhCH$_2$O*C*(=O)–), 172.0 and 173.0 (*C*OOH and –*C*(=O)NHCHCOOH–), 201.5 (–*C*(=O)CH$_2$S$^+$(CH$_3$)$_2$). High-resolution MS (+electrospray): *m*/*z*: found 487.1906; calcd for $C_{25}H_{31}N_2O_6S$, 487.1903.

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