Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 5258

www.rsc.org/obc

PAPER

Site-specific protein propargylation using tissue transglutaminase

Claudio Gnaccarini, Wajih Ben-Tahar, Amina Mulani,† Isabelle Roy, William D. Lubell, Joelle N. Pelletier and Jeffrey W. Keillor†*

Received 18th April 2012, Accepted 15th May 2012
DOI: 10.1039/c2ob25752a

Transglutaminases (TGases) catalyse the transamidation of glutamine residues with primary amines. Herein we report the first FRET-based activity assay for the direct detection of the ligation (transamidation) reaction mediated by tissue TGase (TG2). This novel assay was then used in a microtiter plate-based screen of a library of 18 potential amine substrates. From this screen it was discovered that propargyl amine serves as an excellent substrate for TG2. Subsequently, propargyl amine and 2-azidoethyl amine were validated independently as TG2 substrates with K_M values of 44 ± 4 μM, and 0.99 ± 0.06 mM, respectively. In a proof-of-principle protein labelling experiment, the protein casein was selectively functionalized with propargyl amine using TG2 and subsequently fluorescently labelled through a dipolar cycloaddition reaction with an azido–fluorescein conjugate. This application demonstrates the strong potential of using TG2 for site-specific protein modification through a combination of enzymatic and bioorthogonal chemistry.

Introduction

Transglutaminases (TGases) are Ca^{2+}-dependent enzymes that catalyse the transamidation of peptide- and protein-bound glutamine residues with primary amines, typically the epsilon-amino group of the side-chain of lysine residues. By catalysing the formation of γ-glutamyl-ε-lysine isopeptide bonds, transglutaminases facilitate the cross-linking of proteins,[1,2] which is critical for physiological processes. For example, the stabilisation of blood clots and the extracellular matrix are respectively catalyzed by the TGases Factor XIIIa[3,4] and tissue transglutaminase (TG2).[5]

Proficiency in forming amide bonds using the side-chains of Gln and Lys residues underpins the application of TGases in site-specific protein modification, because unlike sequence specific enzymes, such as subtiligase,[6] sortase,[7] and the split intein system,[8] TGases may be suitable for modifying side chains anywhere within the protein sequence. Recently, lipoic acid ligase has been engineered to mediate acylation of the lysine side chains of a small peptide tag,[9–11] albeit with narrower substrate specificity than TGases for applications such as site-specific protein labelling,[12–14] glycosylation,[15,16] and PEG-ylation.[17]

Paramount for ascertaining the scope and limitations of TGase in such applications is a fundamental understanding of its specificity with respect to acyl-donor and acyl-acceptor substrates. The donor substrate specificity of TGase has been previously studied, confirming the selectivity for glutamine as an acyl-donor substrate selectivity.[18,19] Moreover, features for acyl-donor substrate recognition have been assessed using short peptides and peptide libraries from phage display, demonstrating that certain flanking amino acids enhance recognition of the glutamine residue in high-affinity donor sequences.[20,21]

Less is known about the acyl-acceptor substrates of TGase. For example, TG2 has exhibited selectivity for certain lysine residues in protein sequences, and low specificity for small primary amine substrates.[22] The relevance of flanking amino acids and the global three-dimensional conformation containing the lysine residue remain matters of debate;[23] however, comparisons of amino acid sequences from known protein substrates have led to the design of short peptide substrates, some exhibiting higher affinity than others.[24]

For site-specific protein modification, TG2 has been relatively underexplored, yet exhibits promise, because of its native amino acid acyl-acceptor substrate specificity.[25] Employing a new FRET-based activity assay, which is specific to the transamidation reaction, to screen a proof-of-concept library of amines, several new substrates were identified in this work and applied in TG2-mediated protein labelling. In particular, propargyl amine has been validated as an effective TG2 substrate and employed in a proof-of-principle experiment featuring selective propargylation and dipolar cycloaddition with an azido–fluorescein conjugate to yield a fluorescently labelled protein.

Département de Chimie, Université de Montreal, Succursale centre-ville, C.P. 6128, Montréal, Québec H3C 3J7, Canada
†Current address: Department of Chemistry, University of Ottawa, 10 Marie-Curie, Ottawa, Ontario K1N 6N5 Canada.

Results and discussion

Assay development

A TGase-mediated *ligation* assay was recently reported based on the use of a fluorescent acyl-donor substrate and a biotinylated acyl-acceptor substrate.[26] Seeking a ligation assay that was more amenable to high-throughput screening, direct detection of the ligation reaction has now been achieved without the need for pull down products (Scheme 1). Fluorescein-tagged peptide **1** was employed as acyl-donor substrate.[26] Acyl-acceptor substrate **2**, bearing a primary amine and a complementary fluorophore, was designed to provide a transamidation product exhibiting intramolecular FRET in a continuous fashion, over the course of the reaction when cross-linked to **1** by TGase.

Fluorescent amine **2** was synthesized from cadaverine, which was selectively converted to *N*-(Boc)-1,2-diaminopentane (**4**, Scheme 2). In parallel, coumarin **3** was made from 2,4-dihydroxybenzaldehyde and Meldrum's acid.[27] Coupling of fluorescent acid **3** and amine **4** was accomplished using EDC and HOAt to give carbamate **5**, which was treated with trifluoroacetic acid to remove the Boc group and ion exchanged from trifluoroacetate to chloride, to furnish amine **2**.

The excitation and emission spectra of substrates **1** and **2** were next measured in assay buffer to establish the feasibility of the FRET-based ligation assay (Fig. 1) The significant overlap between the bands for the emission of donor coumarin **2** and the excitation of acceptor fluorescein **1** indicated efficient intramolecular FRET would be achievable under the reaction assay conditions.

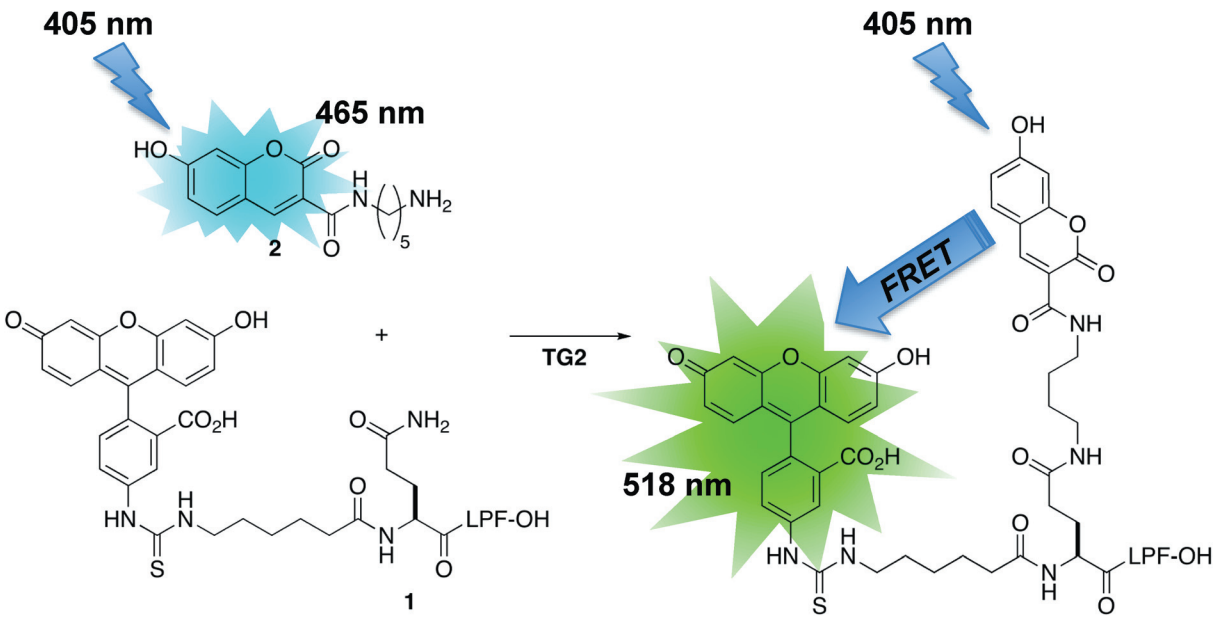
Addition of purified tissue transglutaminase to a solution containing substrates **1** and **2**, caused a proportional change in the intensity of the fluorescence emission bands in a time-dependent manner: the coumarin emission band at 465 nm decreased as the fluorescein emission band at 518 nm increased (Fig. 2). Under the reaction conditions, the relative decrease in coumarin emission was more practical to follow than the relative increase in fluorescein emission.

To the best of our knowledge, this is the first report of a FRET-based activity assay for direct detection of TG2-mediated ligation. Moreover, it is readily amenable to microtiter plate-based applications because as little as 4 mU of purified TG2 gives a detectable decrease in fluorescence at 465 nm. To begin to explore the potential for this assay to detect TG2 activity in a high-throughput fashion under a wide variety of conditions, the FRET-based activity assay was initially employed to screen a proof-of-concept library of potential acyl-acceptor substrates.

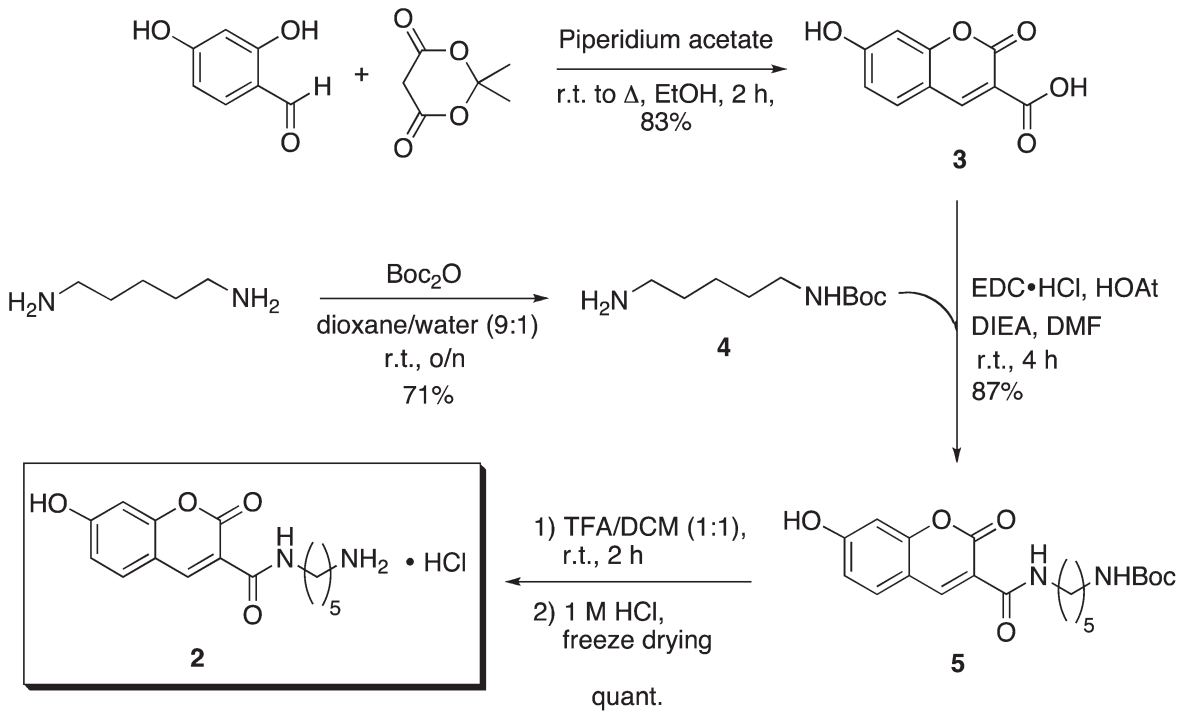
Substrate screening

As we have shown previously,[26] one of the most straightforward applications of an activity assay towards a screening effort is through the design of a simple competition experiment. To that end, 18 different amines were evaluated against amine **2** as acyl-acceptor substrates (Fig. 3). In a microtiter plate, 30 μM of substrates **1** and **2** were initially combined with a different amine candidate in each well at a final concentration of 10 mM and subsequently treated with 60 mU of purified TG2. Amines capable of serving as an effective acceptor substrate competed with amine **2** in the coupling to glutamine donor **1**, and diminished the rate of the decrease of fluorescence at 465 nm, by inhibition of the assay reaction rate. Screens were performed in triplicate and repeated at lower concentrations of competing amine (5 mM final) in triplicate to finally provide a global profile of the relative efficiencies of amines **6–23** as potential acceptor substrates (Fig. 4).

Certain trends in substrate affinity may be inferred from the activities (Fig. 4). For example, unhindered primary amines, such as lysine (**6**), propargyl amine (**13**) and benzyl amine (**17**), all competed effectively with substrate **2**. Adjacent carboxylate



Scheme 1



Scheme 2

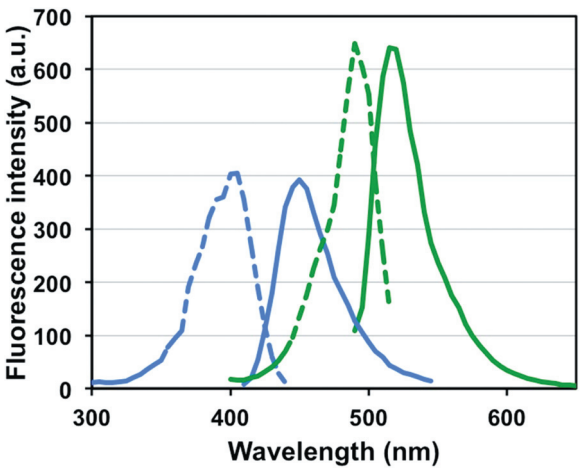


Fig. 1 Excitation (dashed) and emission (solid) bands of equimolar solutions of substrates **2** (400/450 nm) and **1** (490/520 nm).

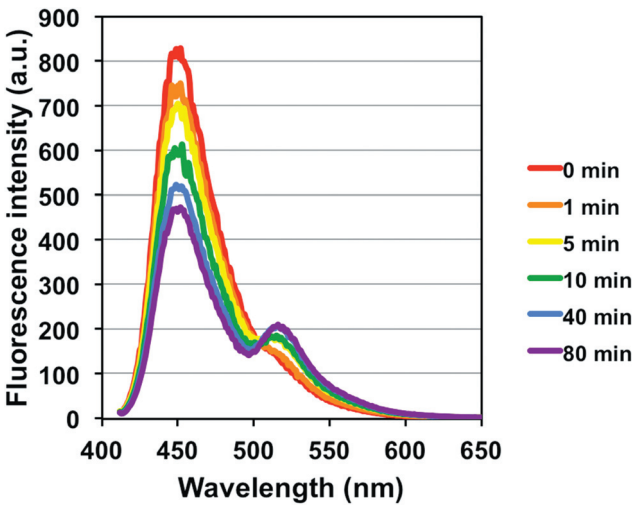


Fig. 2 Time-dependent decrease of coumarin emission (~465 nm) and concomitant increase in fluorescein emission (~518 nm), due to trans-glutaminase-mediated ligation of equivalent concentrations of substrates **1** and **2**.

moieties reduced efficacy [*e.g.* 6-aminohexanoic acid (**7**), γ-aminobutyric acid (**8**, GABA), alanine (**10**), and *p*-aminomethylbenzoic acid (**19**)], which could be restored by esterification as in the cases of ethyl 3-aminopropionate (**9**) and alanine methyl ester (**11**). Anilines **20–23** were relatively less active than the benzyl amines, perhaps owing to their decreased nucleophilicity, and the presence of a carboxylate moiety in *p*-aminophenylacetic acid (**23**) diminished activity. The observation that propargyl amine (**13**) served as an efficient substrate for TG2 opened the door to site-specific protein modification by enzymatic propargylation followed by dipolar cycloadditions with azides (*vide infra*).

TG2-mediated protein labelling

The discovery of propargyl amine (**13**) as an effective substrate inspired investigation of the site-specific transamidation of glutamine residues with TG2 followed by conjugation with an appropriate label by azide–alkyne cycloaddition. In addition to propargyl amine (**13**), 2-azidoethylamine was examined as substrate for TG2 in order to develop a complementary protein labelling process. 2-Azidoethylamine (**25**) was synthesized in three steps from 2-bromoethylamine by amine protection with

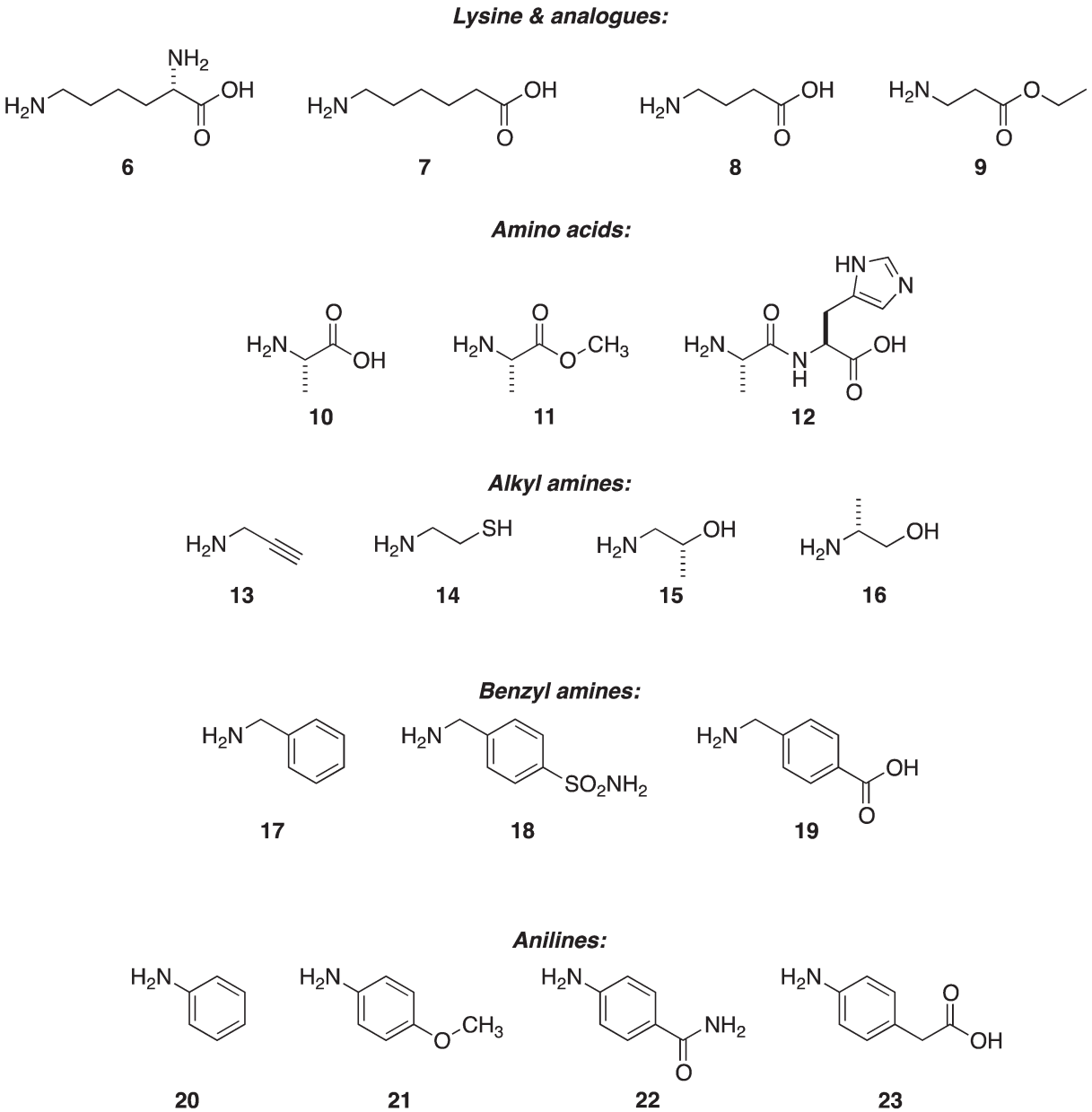


Fig. 3 Amines **6–23** screened as potential acyl-acceptor TG2 substrates.

di-*tert*-butyldicarbonate, displacement of bromide with sodium azide and carbamate cleavage with HCl (Scheme 3).

It is possible, although improbable, that a positive result from our indirect competition assay may derive from inhibition of the assay reaction by the competing amine, without that amine serving as a genuine substrate. Therefore, we deemed it critical to evaluate the most important putative amine substrates by independent assay methods. Using a known chromogenic activity assay,[22] the k_{cat} and K_M values for propargyl amine (**13**) were thus determined to be 2.39 ± 0.04 min^{-1} and 44 ± 4 μM, respectively, whereas for 2-azidoethylamine (**25**) they were found to be 2.09 ± 0.04 min^{-1} and 0.99 ± 0.06 mM. In subsequent applications, amine **13** was employed, because of its greater affinity (K_M) and efficiency (k_{cat}/K_M). Amine **13** was furthermore reacted with the glutamine residue of the heptapeptide Ac-PNPQLPF, which was prepared by solid-phase synthesis (see Experimental) and had previously exhibited high affinity for TG2 with an apparent K_M of 11 ± 1 μM in competition[28] with a chromogenic assay.[22] The heptapeptide was reacted with propargyl amine for two hours in the presence of TG2 at 37 °C. Product formation was observed as the appearance of a new peak with corresponding mass in the LC-MS chromatogram.

Proof-of-concept site-specific labelling with propargyl amine was then performed using dimethyl casein, a known protein donor substrate of TG2,[29] followed by cycloaddition on the alkyne employing fluorescein azide **26**. Azide **26** was prepared by acylation of amino azide **25** with FITC (Scheme 3). Dimethyl casein was reacted with one equivalent of propargyl amine at a final concentration >10 times of its K_M value in the presence of TG2. After two hours, the reaction mixture was treated with

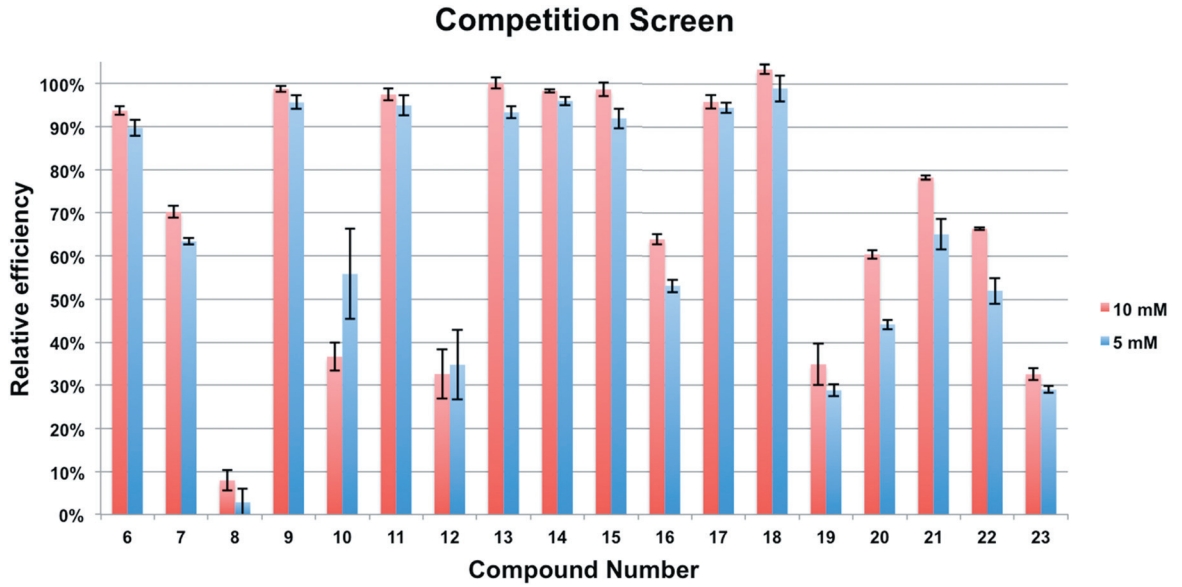
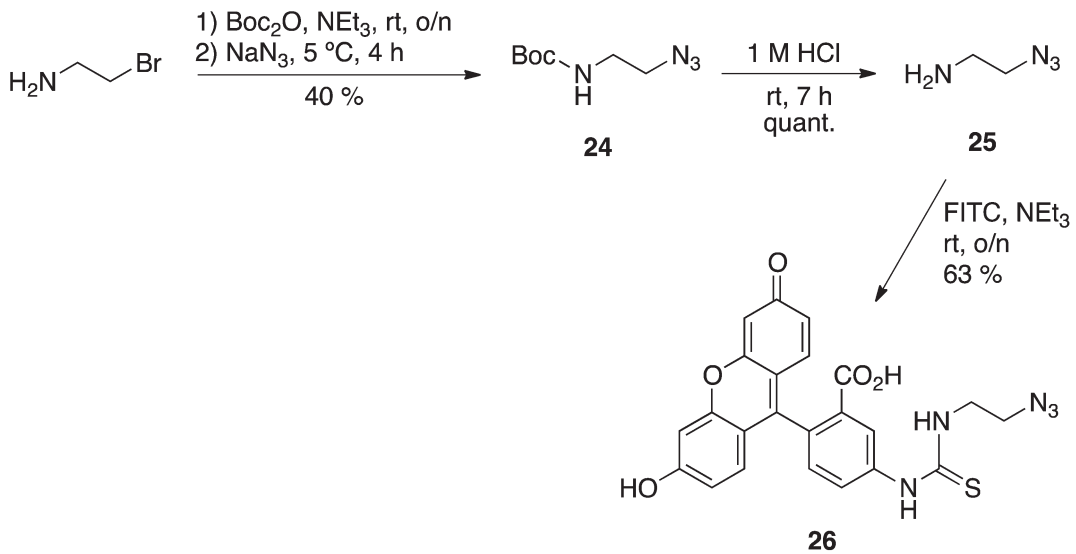


Fig. 4 Relative efficiency of amines **6–23** (Fig. 3) in competition with fluorescent amine **2** (Scheme 1). Activity was measured at final concentrations of 5 and 10 mM of competing amine and error bars represent standard deviations from screening results obtained in triplicate.



Scheme 3

azide **26**, copper and ascorbic acid[30] and allowed to sit overnight, prior to analysis by SDS–PAGE (Fig. 5). Both bands of casein were efficiently fluorescently labelled by this procedure; however, in control experiments without TG2 no labelling was observed.

Summary

A new assay for studying TG2 transpeptidation activity has been developed featuring ligation of fluorescently labelled substrates and detection of intramolecular FRET. This assay is specific for transpeptidation, sensitive, and suitable for high-throughput applications. Using this assay, a library of 18 amines was screened in competition with the fluorescent amine substrate to provide a structure–activity profile for acyl-acceptor substrates of TG2. From this screen, propargyl amine was discovered to be an effective TG2 substrate, as confirmed by independent assays, and subsequently employed in a proof-of-principle protein labelling experiment featuring a transamidation/cycloaddition sequence. Considering the potential of TG2 to site-specifically propargylate diverse proteins, including those modified with high-affinity glutamine sequences,[31] our results may open many doors for various applications, featuring modification of the propargylated proteins using a spectrum of different azide tags. In addition, the complementary modification of azide bearing proteins with different alkyne derivatives[11,32] may be performed, because we have also shown that TG2 can recognize

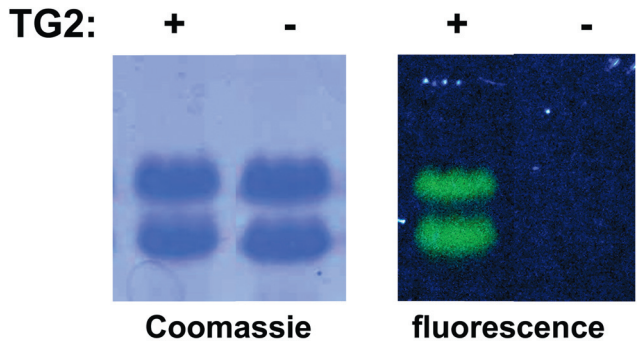


Fig. 5 Protein labelling with fluorescent tag by TG2-mediated propargylation of casein, followed by azide cycloaddition: in the absence of TG2, no labelling occurs.

2-azidoethyl amine as a substrate. These protein-labelling methods take advantage of the site-specificity and orthogonality of TG2-mediated transamidation, as well as the chemical diversity of subsequent cycloaddition reactions, rather than attempting to use a wide variety of labelling agents as substrates for TG2. TG2-based modifications have thus been established as effective methods for labelling proteins.

Experimental section

Materials

Synthesis generalities. All Fmoc-protected amino acids, resins and coupling reagents were purchased from GL Biochem; Wang resin was purchased from NovaBiochem. All other reagents were obtained from Sigma-Aldrich. Reactions requiring anhydrous conditions were carried out under a dry nitrogen atmosphere employing conventional bench-top techniques. ^{1}H- and ^{13}C-NMR spectra were recorded on AMXR400 and AMX300 spectrometers and were referenced to the residual proton or ^{13}C signal of the solvent. Mass spectra were determined by FAB+ ionization on an AutoSpec Q spectrometer at the Regional Mass Spectrometry Centre at the Université de Montréal. Reactor tubes for solid-phase peptide synthesis were obtained from Supelco. All resins were swollen in DMF and washing steps were performed using CH_2Cl_2 and DMF (EMD Chemicals).

Synthesis of the FRET donor substrate

7-Hydroxycoumarin-3-carboxylic acid ***(3)****.* Preparation of coumarin **3** was based on a literature procedure.[27] A mixture of 2,4-dihydroxyl benzaldehyde (2 g, 20 mmol), Meldrum's acid (2.89 g, 20 mmol), piperidinium acetate (58 mg, 0.4 mmol) and ethanol (10 mL) was stirred at room temperature for 20 min, heated at reflux for 2 h, allowed to cool to room temperature and chilled in an ice bath for 1 h. The crystallized product was filtered, washed three times with ethanol, and dried *in vacuo* yielding **3** (3.4 g, 83%) as an off-white powder; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.66 (s, 1H), 7.73 (d, 1H, J = 8.5), 6.84 (d, 1H, J = 8.4), 6.73 (s, 1H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 164.3, 164.1, 157.7, 157.1, 149.3, 132.1, 114.1, 112.7, 110.7, 101.9.

(5-Aminopentyl)carbamic acid tert*-butyl ester* ***(4)****.* A solution of di-*tert*-butyldicarbonate (2.13 g, 9.8 mmol) in 40 mL of 9 : 1 dioxane–water was added over a period of 4 h to a solution of 1,5-diaminopentane (2 g, 19.6 mmol) in 60 mL of 9 : 1 dioxane–water. The solution was stirred at room temperature overnight and concentrated by rotary evaporation. The residue was dissolved in 40 mL of water. The white precipitate corresponding to *N*,*N*′-di-Boc-1,5-diaminopentane was removed by filtration through a fritted glass funnel, and the filtrate was extracted with CH_2Cl_2 (4 × 30 mL). The combined organic extracts were concentrated. The residue was purified by silica gel chromatography (90 : 10 CH_2Cl_2–methanol). Evaporation of the collected fractions provided *N*-(Boc)-1,5-diaminopentane (**4**, 1.4 g, 6.95 mmol, 71%) as a dense liquid: bp 97 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.63 (s, 1H), 3.1 (dd, 2H, J = 6.0, J = 12.4), 2.68 (t, 2H, J = 6.7), 1.75 (s, 3H), 1.49–1.52 (br, 3H), 1.42 (s, 9H), 1.27–1.36 (br, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 156.9, 79.9, 42.8, 41.3, 34.0, 30.7, 29.2, 24.9. HRMS m/z (M + H$^+$): calcd for $C_{10}H_{23}N_2O_2$ 203.1754; found 203.1761.

{5-[(7-Hydroxy-2-oxo-2H-chromene-3-carbonyl)amino]pentyl}-carbamic acid tert*-butyl ester* ***(5)****.* A solution of acid **3** (30 mg, 0.15 mmol) in 3 mL of DMF was treated with amine **4** (31 mg, 0.15 mmol), HOAt (21 mg, 0.15 mmol), EDC·HCl (29 mg, 0.15 mmol) and DIEA (0.027 mL, 0.15 mmol) and stirred at room temperature for 4 h, when TLC showed the disappearance of the starting amine (R_f = 0, solvent: 5% MeOH–95% AcOEt) and appearance of a new less polar spot (R_f = 0.2, solvent: 5% MeOH–95% AcOEt). The volatiles were removed by rotary evaporation and the residue was treated with 5 mL of MeOH, leading to formation of a white precipitate. This precipitate was recovered by filtration onto a fritted funnel, washed with 3 × 2 mL of MeOH, and dried under vacuum overnight yielding amide **5** (50.1 mg, 0.13 mmol, 87%) as white solid: mp 176.3 °C; ^{1}H-NMR (DMSO d_6, 400 MHz) δ 11.16 (s, 1H), 8.77 (s, 1H), 8.61 (t, 1H, J = 5.6), 7.81 (d, 1H, J = 8.6), 6.88 (dd, 1H, J = 8.6, J = 2.2), 6.8 (d, 1H, J = 2.1), 6.73 (br, 1H), 3.18 (d, 1H, J = 4.8), 2.91 (q, 2H, J = 12.8, J = 6.6), 1.51 (m, 2H), 1.36 (s, 9H), 1.28 (br, 3H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 164.9, 162.3, 156.8, 155.2, 149.1, 134.8, 130.6, 126.1, 119.5, 119.4, 117.4, 79.9, 41.3, 40.5, 30.5, 30.0, 29.2, 25.0. HRMS m/z (M + Na$^+$): calcd for $C_{20}H_{26}N_2O_6$ 413.1683; found 413.1695.

7-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid (5-amino-pentyl)-amide ***(2)****.* A solution of carbamate **5** (20 mg, 0.06 mmol) in a mixture of TFA–DCM (1 mL/1 mL) was stirred for 2 h. The volatiles were removed under vacuum. A salt exchange was performed by dissolving the residue in 2 mL of 1 M HCl and freeze-drying, twice. Amine hydrochloride **2** was isolated as a yellow gel in quantitative yield (19.3 mg, 0.06 mmol); λ_{ex} = 400 nm, λ_{em} = 448 nm. ^{1}H NMR (CD_3OD, 400 MHz) δ: 9.63 (s, 1H), 8.63 (d, 1H, J = 8.80), 7.88 (d, 1H, J = 8.80), 7.77 (s, 1H), 4.36 (t, 2H, J = 6.60), 3.94 (t, 2H, J = 7.74), 2.56–2.71 (m, 4H), 2.35–2.46 (m, 2H). HRMS m/z (M + H$^+$): calcd for $C_{15}H_{19}N_2O_4$ 291.1341; found 291.1339.

Synthesis of azide substrate

tert*-Butyl 2-azidoethylcarbamate* ***(24)****.* A solution of 2-bromoethylamine hydrobromide (5 g, 24.4 mmol) in 20 mL of MeOH was treated with 3 mL of triethylamine and 10 g (45.8 mmol) of di-*tert*-butyldicarbonate, stirred at room temperature overnight, treated with another 5 g (22.9 mmol) of di-*tert*-butyldicarbonate,

and stirred for 75 min. Without isolation, the carbamate solution was cooled to 5 °C, treated with 4.8 g (73.2 mmol) of sodium azide and 20 mL of H_2O and stirred for 4 h at 5 °C. The reaction mixture volume was reduced by evaporation, and treated with 40 mL of water. The pH was adjusted to 4 using 1 M HCl. The reaction mixture was extracted with 40 mL of CH_2Cl_2, followed by 3 × 20 mL of CH_2Cl_2. The combined organic phases were dried on $MgSO_4$, filtered and evaporated on a rotary evaporator to yield 1.8 g of azide **24** (9.7 mmol, 40% yield). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.40 (s, 9H), 3.25 (m, 2H), 3.36 (t, J = 5.39 Hz, 2H) 4.84 (br s, 1H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 15.0, 29.1, 40.9, 52.0, 80.6, 156.56. HRMS m/z ($M + Na^+$): calcd for $C_7H_{14}N_4O_2Na$ 209.1009; found: 209.1009.

*2-Azidoethylamine (**25**).* A solution of 200 mg (1.1 mmol) of carbamate **24** in 3 mL MeOH was treated with 4 mL of 1 M HCl and stirred at room temperature for 7 h. The volume was reduced by rotary evaporation, diluted with 3 mL of water and freeze-dried overnight, giving quantitatively 131.6 mg (1.1 mmol) of **25** as its hydrochloride salt. ^{1}H-NMR (CD_3OD, 400 MHz) δ: 3.14 (br t, 2H), 3.75 (br t, J = 5.65 Hz, 2H). ^{13}C-NMR (CD_3OD, 100 MHz) δ: 39.4, 48.9. HRMS m/z ($M + H)^+$ calcd for $C_2H_7N_4$: 87.0665; found: 87.0662.

*5-(3-(2-Azidoethyl)thioureido)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-benzoic acid (**31**).* A solution of fluorescein isothiocyanate (300 mg, 0.77 mmol) in 30 mL of MeOH was treated with 1 mL of Et_3N and 131 mg of 2-azidoethyl amine (**25**, 1.07 mmol), stirred overnight, and evaporated to a residue that was purified by silica gel chromatography using ethyl acetate–methanol–acetic acid 15 : 1 : 0.1 as eluant. Evaporation of the collected fractions gave 230 mg (0.48 mmol) of azide **26** (63% yield). ^{1}H-NMR (CD_3OD, 400 MHz) δ: 3.53 (t, J = 5.77, 2H), 3.75 (t, J = 5.77, 2H), 6.47–6.64 (m, 6H), 7.10 (d, J = 8.24, 1H), 7.70 (dd, J = 8.24, J = 1.90, 1H), 8.10 (d, J = 1.90, 1H). ^{13}C-NMR (CD_3OD, 100 MHz) δ: 44.2, 50.6, 103.0, 110.9, 113.1, 119.7, 125.2, 128.5, 129.8, 131.6, 141.6, 149.2, 153.6, 161.0, 170.6, 174.8, 182.8. HRMS m/z ($M + H^+$) calcd for $C_{23}H_{18}N_5O_5S$: 476.1016; found: 476.10232.

Synthesis of peptide substrate

Ac-PNPQLPF. The peptide Ac-PNPQLPF was synthesized using standard solid-phase Fmoc chemistry.[33] Briefly, the first Fmoc protected amino acid (5 mmol) was coupled to Wang resin (1.1 mmol) using DIC (5 mmol) and DMAP (0.1 mmol) in DMF (5 resin volumes). The level of loading of the amino acid on the resin after the first coupling step was determined by spectroscopic measurement of the UV absorbance of the piperidine dibenzofulvalene adduct formed during Fmoc deprotection. This loading level was used as the resin loading capacity for all subsequent steps. The remaining free hydroxyl functionalities were capped by shaking the resin for 2 h in a mixture of acetic anhydride–pyridine (2 : 3). After washing with DMF (three times with 10 resin volumes), DCM (three times with 10 resin volumes), and ether (three times with 10 resin volumes), the Fmoc group was removed by incubating with piperidine in DMF (20% v/v; 10 resin volumes) for 1 h, followed by washing with DMF (three times with 10 resin volumes), DCM (three times with 10 resin volumes), and ether (three times with 10 resin volumes) in preparation for the next amide coupling. Deprotection was qualitatively verified by a positive Kaiser test on a sample of a few beads. Subsequently, each Fmoc protected amino acid (1.6 mmol) was coupled to Wang resin preloaded with the necessary carboxyl-terminal amino acid (0.63 mmol) in DMF (5 resin volumes) using HOBt (1.6 mmol) and DIC (1.6 mmol). Couplings were performed twice for 30 min and verified by a negative Kaiser test on a sample of a few beads. Final acetylation was performed by shaking the resin for 15 min with a mixture of acetic anhydride and triethylamine in DMF (2.5/0.75/25, 10 resin volumes). Acetylation was verified by a negative Kaiser test on a sample of a few beads.[34] The final peptide was cleaved from the resin (1 g) by incubating with TFA–DCM (1 : 1) over 2 h. The peptide was precipitated from the cleavage solution using diethyl ether and hexane, and the crude peptide was purified by preparative HPLC, using a Synergi Polar-RP, 100 × 21.20 mm column (Phenomenex, Torrance, CA) on a Varian (Prep Star) HPLC system.

Enzyme purification. Recombinant guinea pig liver transglutaminase (TG2) was expressed and purified according to a literature procedure.[35] In brief, *N*-terminal His-tagged guinea pig liver transglutaminase was co-expressed with the DnaK/DnaJ chaperone protein[36] from co-transformed XL1-Blue *E. coli* cells, induced overnight in the presence of 1 mM IPTG at 28 °C. After lysis of the bacterial cells by sonication, the overexpressed His-tagged TG2 was purified by affinity chromatography on Ni-NTA agarose, followed by desalting.

Methods

Ligation activity assay and competition screen. In a 96-well microtiter plate, each well was charged with 10 µL of 0.6 mM fluorescein amide substrate **1**, 10 µL of 0.6 mM coumarin amine substrate **2**, and 80 µL buffer (200 mM Tris HCl (pH 8), 10 mM $CaCl_2$, 1.2 mM EDTA), and treated subsequently with either 10 or 20 µL of a 100 mM stock solution of the amine candidate (**6–23**), followed by 90 or 80 µL of water to give final concentrations of 5 or 10 mM, respectively, in a final volume at 200 µL. In control runs, 100 µL water was added instead of the amine solution. Reactions were initiated by adding 20 µL of a 3 U mL^{-1} stock solution of purified TG2. In control experiments, the enzyme storage buffer [25 mM Tris acetate (pH 7)] was used instead of TG2 solution. The time-dependent decrease in fluorescence was followed using a 465 nm emission filter.

Peptide propargylation. A 1.5 mL Eppendorf tube was charged with 10 µL of a 66 U mL^{-1} TG2 solution, 5 µL of a propargyl amine stock solution (100 mM in buffer), 50 µL of a solution of peptide Ac-PNPQLPF (11.7 mM in DMF) and 935 µL of buffer (200 mM MOPS (pH 7), 0.1 M $CaCl_2$, 20 mM EDTA). After 2 h at 37 °C, to the mixture was centrifuged over an Amicon Centrifugal Filter Unit (30 kDa MWCO) at 4 °C for 30 min at 1962*g*. The filtrate was collected and characterized by LC-MS, whereby a new peak was detected, whose mass was 892.4, corresponding to the expected product [Ac-PNPQ(*N*-propargyl)LPF]$^+$.

Protein labelling. To 1 mL of a 12 mg mL^{-1} (0.5 mM) solution of dimethyl casein in buffer (100 mM Tris HCl (pH 8), 5 mM $CaCl_2$), 5 µL of 100 mM propargyl amine and 40 µL of 18 mg mL^{-1} purified TG2 were added and the reaction was

mixed gently for 2 h at room temperature. As a negative control the same reaction was prepared without adding TG2. The TG2 and control reaction mixtures were treated with 4 μL of 50 mM $CuCl_2$, 20 μL of 50 mM ascorbic acid, 10 μL of 50 mM FITC-azide **26**, and 12 μL of 50 mM 1,2-phenylene diamine, and mixed gently overnight at 4 °C. The reaction products were subsequently analysed by 10% SDS–PAGE, separating the two casein proteins present in the commercial product. The fluorescence of these bands was recorded prior to Coomassie staining.

References

1 L. Lorand and R. M. Graham, *Nat. Rev. Mol. Cell Biol.*, 2003, **4**, 140–156.
2 M. Griffin, R. Casadio and C. M. Bergamini, *Biochem. J.*, 2002, **368**, 377–96.
3 L. Muszbek, V. C. Yee and Z. Hevessy, *Thromb. Res.*, 1999, **94**, 271–305.
4 L. Lorand, *J. Thromb. Haemostasis*, 2005, **3**, 1337–1348.
5 D. Aeschlimann and V. Thomazy, *Connect. Tissue Res.*, 2000, **41**, 1–27.
6 T. K. Chang, D. Y. Jackson, J. P. Burnier and J. A. Wells, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 12544–12548.
7 H. Mao, S. A. Hart, A. Schink and B. A. Pollock, *J. Am. Chem. Soc.*, 2004, **126**, 2670–2671.
8 H. Wu, Z. Hu and X. Lui, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 9226–9231.
9 C. Uttamapinant, K. A. White, H. Baruah, S. Thompson, M. Fernandez-Suarez, S. Puthenveetil and A. Y. Ting, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 10914–10919.
10 J. D. Cohen, S. Thompson and A. Y. Ting, *Biochemistry*, 2011, **50**, 8221–8225.
11 J. Z. Yao, C. Uttamapinant, A. Poloukhtine, J. M. Baskin, J. A. Codelli, E. M. Sletten, C. R. Bertozzi, V. V. Popik and A. Y. Ting, *J. Am. Chem. Soc.*, 2012, **134**, 3720–3728.
12 U. Bechtold, J. T. Otterbach, R. Pasternack and H.-L. Fuchsbauer, *J. Biochem.*, 2000, **127**, 239–245.
13 J.-H. Jeon, C.-W. Kim, D.-M. Shin, K.-I. Kim, S.-Y. Cho, J.-C. Kwon, K.-H. Choi, H.-S. Kang and I.-G. Kim, *FEBS Lett.*, 2003, **534**, 180–184.
14 M. Jäger, E. Nir and S. Weiss, *Protein Sci.*, 2006, **15**, 640–646.
15 S.-C. B. Yan and F. Wold, *Biochemistry*, 1984, **23**, 3759–3765.
16 A. Valdivia, R. Villalonga, P. Di Pierro, Y. Pérez, L. Mariniello, L. Gòmez and R. Porta, *J. Biotechnol.*, 2006, **122**, 326–333.
17 H. Sato, M. Ikeda, K. Suzuki and K. Hirayama, *Biochemistry*, 1996, **35**, 13072–13080.
18 L. Lorand and S. M. Conrad, *Mol. Cell. Biochem.*, 1984, **58**, 9–35.
19 J. E. Folk, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1983, **54**, 1–56.
20 Z. Keresztessy, E. Csosz, J. Hársfalvi, K. Csomós, J. Gray, R. N. Lightowlers, J. H. Lakey, Z. Balajthy and L. Fésüs, *Protein Sci.*, 2006, **15**, 2466–2480.
21 Y. Sugimura, M. Hosono, F. Wada, T. Yoshimura, M. Maki and K. Hitomi, *J. Biol. Chem.*, 2006, **281**, 17699–17706.
22 A. Leblanc, C. Gravel, J. Labelle and J. W. Keillor, *Biochemistry*, 2001, **40**, 8335–8342.
23 S. N. P. Murthy, T. J. Lukas, T. S. Jardetzky and L. Lorand, *Biochemistry*, 2009, **48**, 2654–2660.
24 B.-H. Hu and P. B. Messersmith, *J. Am. Chem. Soc.*, 2003, **125**, 14298–14299.
25 J. W. Keillor, R. A. Chica, N. Chabot, V. Vinci, C. Pardin, E. Fortin, S. M. F. G. Gillet, Y. Nakano, M. T. Kaartinen, J. N. Pelletier and W. D. Lubell, *Can. J. Chem.*, 2008, **86**, 271–276.
26 C. Gnaccarini, W. Ben-Tahar, W. D. Lubell, J. N. Pelletier and J. W. Keillor, *Bioorg. Med. Chem.*, 2009, **17**, 6354–6359.
27 A. Song, X. Wang and K. S. Lam, *Tetrahedron Lett.*, 2003, **44**, 1755–1758.
28 R. Castonguay, C. Lherbet and J. W. Keillor, *Bioorg. Med. Chem.*, 2002, **10**, 4185–4191.
29 A. Case and R. L. Stein, *Biochemistry*, 2003, **42**, 9466–9481.
30 M. Meldal and C. W. Tornøe, *Chem. Rev.*, 2008, **108**, 2952–3015.
31 C.-W. Lin and A. Y. Ting, *J. Am. Chem. Soc.*, 2006, **128**, 4542–4543.
32 J. Tummatorn, P. Batsomboon, R. J. Clark, I. V. Alabugin and G. B. Dudley, *J. Org. Chem.*, 2012, **77**, 2093–2097.
33 W. D. Lubell, J. W. Blankenship, G. Fridkin and R. Kaul, *Science of Synthesis*, Thieme, Stuttgart, 2005, ch. 21.11, pp. 713–809.
34 G. W. Tregear and J. T. Potts Jr., *Endocr. Res.*, 1975, **2**, 561–570.
35 S. M. F. G. Gillet, R. A. Chica, J. W. Keillor and J. N. Pelletier, *Protein Expression Purif.*, 2004, **33**, 256–264.
36 K.-I. Yokoyama, Y. Kikuchi and H. Yasueda, *Biosci., Biotechnol., Biochem.*, 1998, **62**, 1205–1210.