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Development of a dual fluorogenic and chromogenic dipeptidyl peptidase IV substrate

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Abstract—A new far-red dual fluorogenic and chromogenic substrate, 5-glycylprolylglycylprolyl-9-di-3-sulfonyl-propylaminobenza[*a*]phenoxazonium perchlorate (GPGP-2SBPO), was developed for dipeptidyl peptidase IV (DPP-IV) sensing. The glycylprolylglycylprolyl tetrapeptide was chosen as the recognition sequence due to its stability under physiological conditions. In contrast, the truncated substrate, GP-2SBPO, containing only a glycylprolyl peptide, is unstable. Proteolysis of GPGP-2SBPO was assayed by monitoring the absorbance and fluorescence signals from the released fluorochrome, 2SBPO, at 625 and 670 nm, respectively.

Dipeptidyl peptidase IV (DPP-IV) is an enzyme of considerable biomedical interest because it is upregulated in certain diseases.[1–3] DPP-IV is a serine protease, which cleaves Xxx-Pro dipeptides from the N-termini of polypeptides and proteins with a penultimate proline residue. In vitro, the activity of DPP-IV is often assayed using chromogenic substrates, such as glycylproline β-naphthylamide[4] and glycylproline *p*-nitroanilide,[5] or fluorogenic probes, such as $(Ala\text{-}Pro)_2$-cresyl violet and $(Ala\text{-}Pro)_2$-Rhod110.[6] Although these probes are commonly available, they have poor aqueous solubility and frequently require organic solvents such as DMSO for solubilization. The use of organic co-solvents in enzymatic assays for DPP-IV may lead to unreliable results. For example, DPP-IV activity has been reported to be modulated by DMSO, with 1% DMSO reducing activity by 50%.[7] Moreover, both $(Ala\text{-}Pro)_2$-cresyl violet and $(Ala\text{-}Pro)_2$-Rhod110 are unstable in aqueous solution and undergo hydrolysis in the absence of DPP-IV.[6] Therefore, fluorogenic $(Ala\text{-}Pro)_2$-cresyl violet and $(Ala\text{-}Pro)_2$-Rhod110 are of limited use for sensing DPP-IV activity in live cells[8,6] or in vivo. There is thus an urgent need for the development of improved probes which are stable in aqueous solution, are water-soluble, have far-red or near-IR excitation and emission maxima for improved tissue penetration and have low background in biological samples. For in vivo use, the ideal fluorochromes would have emission maxima between 650 and 900 nm.[9]

Recently, we reported a new type of water-soluble far-red fluorogenic molecule containing a disulfonated benzo[*a*]phenoxazine (2SBPO) scaffold.[10] Peptide conjugates of this fluorochrome are weakly fluorescent but display strong fluorescence emission at 670 nm following proteolytic release of the fluorochrome. In addition, dramatic changes in absorbance at 625 nm are observed. In the present report, the synthesis and preliminary biochemical studies of a DPP-IV sensitive probe based on the above scaffold are detailed.

All amino acids were purchased from Novabiochem (San Diego, CA). The parent dye 9-di-3-sulfonyl-propylaminobenzo[*a*]phenoxazonium perchlorate (2SBPO), synthesized as previously described,[10] was adjusted to pH 8.5 and lyophilized prior to use. Dipeptidyl aminopeptidase (DPP-IV, EC 3.4.14.5, from porcine kidney) and other common reagents were obtained from Sigma Aldrich (St. Louis, MO) and used without further purification. Absorbance spectra were measured on a Varian Cary 50-Bio UV/Visible spectrophotometer (Palo Alto, CA). Fluorescence spectra were carried out on a F-4500 spectrofluorometer (Hitachi, Danbury, CT). Fluorescence measurements for stability studies and enzyme kinetics were determined on a monochromator-based microplate detection system (Safire2, TECAN,

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San Jose, CA) with excitation/emission band width of 20/20 nm. MALDI-TOF mass spectra were measured on a Voyager linear mass spectrometer (PE Biosystems; Framingham, MA). The kinetic parameters (K_m and V_{max}) were determined using the direct linear plot of Eisenthal and Cornish-Bowden.

Synthesis of Gly-Pro-2SBPO (GP-2SBPO). To a stirred solution of *N*-α-*t*-Boc-L-proline (21 mg, 0.1 mmol) in DMF (0.8 mL) were added 2SBPO (20 mg, 0.033 mmol), *N*-hydroxybenzotriazole (HOBT) (37 mg, 0.1 mmol), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (14 mg, 0.1 mmol), and *N*,*N*-diisopropylethylamine (DIPEA) (26 mg, 0.2 mmol). The solution was stirred at room temperature for 1 h and monitored by silica gel TLC. When the reaction was complete, the crude product was precipitated with diethyl ether and the precipitate was treated with TFA (1 mL) to remove the Boc-protecting group. After 15 min, diethyl ether was added and the precipitate was collected. The crude product was then coupled with Boc-glycine using the previous coupling conditions. The product was precipitated with diethyl ether and purified on a 10 g RP C18 cartridge (Waters) eluting with 20% acetonitrile in water to give GP-2SBPO as a blue solid (22 mg, 91%). MS (MALDI-TOF), calcd for $C_{29}H_{35}N_5O_9S_2$ $(M+H)^+$: 661.28. Found: 661.75.

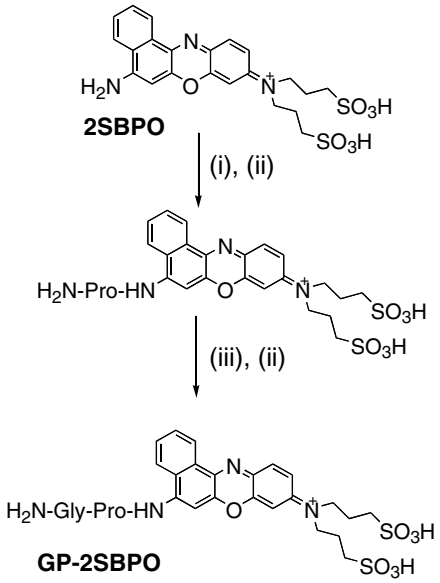
Synthesis of Gly-Pro-Gly-Pro-2SBPO (GPGP-2SBPO). GPGP-2SBPO was synthesized and purified using the same procedure as described for GP-2SBPO. Overall yield was 90%. MS (MALDI-TOF), calcd for $C_{36}H_{45}N_7O_{11}S_2$ $(M+H)^+$: 815.49. Found: 815.59. The molar extinction coefficient of GPGP-2SBPO at 630 nm is 7000 $(M\ cm)^{-1}$ in 0.1 M PBS buffer.

Aqueous stability of GPGP-2SBPO and GP-2SBPO. Stock solutions of compounds GPGP-2SBPO and GP-2SBPO were prepared in water at a concentration of 1×10^{-4} M. The stock solutions were then added to 0.1 M, pH 4.0–8, Tris buffers. The final concentration of the solutions was 1×10^{-6} M. The fluorescence emission at 670 nm was measured with excitation at 630 nm using a plate reader for 3 h.

Proteolysis of GPGP-2SBPO by DPP-IV. A solution of DPP-IV (10 μL, 18 mU) was added to GPGP-2SBPO (200 μL, 25 μM) in 0.1 M HEPES buffer (pH 7.4) containing 140 mM NaCl, 10 mM KCl, and 0.1% bovine serum albumin at 37 °C. The increase in fluorescence emission at 670 nm was measured using a fluorescence plate reader with excitation at 630 nm. The absorption increases at 625 nm were measured using a UV–vis spectrophotometer from $t = 0$ to 100 min. The same conditions were applied to the control sample solution without adding DPP-IV.

Water-soluble 2SBPO was chosen for development of the dual fluorogenic and chromogenic substrates because of its superior optical properties in aqueous buffer as previously reported.[10] Since glycylprolyl containing peptide substrates for DPP-IV are in common use, GP-2SBPO was initially synthesized as a potential fluorogenic substrate for DPP-IV (Scheme 1). Boc-protected amino acids were activated in situ by HBTU and coupled sequentially to 2SBPO using diisopropylethylamine as base. The coupling reactions were typically complete in 1 h at room temperature with near quantitative conversion as determined by TLC. The final products were purified by RP C18 chromatography.

As expected, the synthesized dipeptide substrate, GP-2SBPO, was only weakly fluorescent. Initial studies with DPP-IV under the physiological buffer conditions showed high fluorescent signal after incubation. However, when the substrate was incubated under identical conditions in the absence of DPP-IV, a similar increase in fluorescence was also detected. Therefore, the stability of GP-2SBPO in aqueous solution was systematically examined over the pH range from 4 to 8 in 0.1 M Tris buffers. After 3 h of incubation at 27 °C, GP-2SBPO was found only to be stable below pH 5; when the pH was above 6, probe hydrolysis was significant (Fig. 1).



Scheme 1. Synthetic scheme of GP-2SBPO. Reagents and conditions: (i) 3 equiv Boc-Pro-OH, 3 equiv HOBT/HBTU, 6 equiv DIPEA/DMF; (ii) TFA; (iii) 3 equiv Boc-Gly-OH, 3 equiv HOBT/HBTU, 6 equiv DIPEA/DMF.

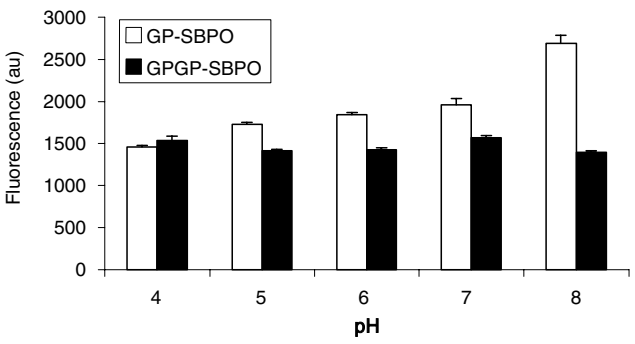
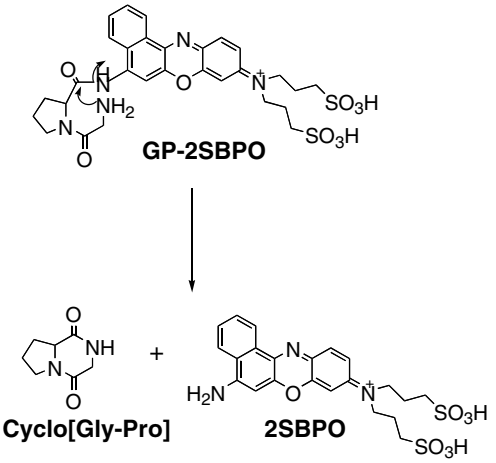


Figure 1. Effect of pH on the stability of GP-2SBPO and GPGP-2SBPO in 0.1 M Tris buffer.

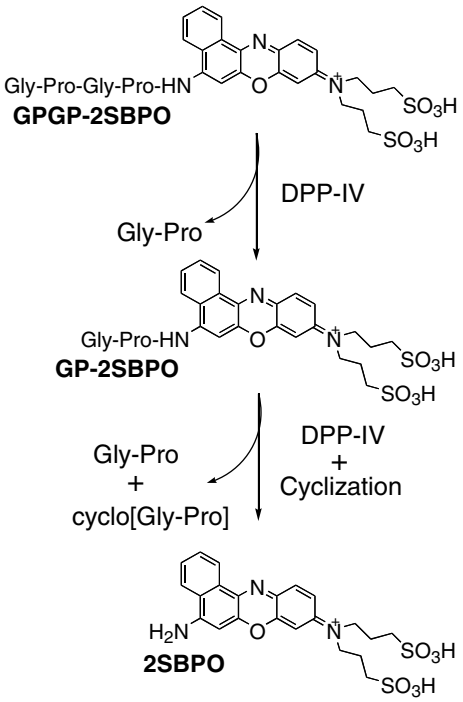
One possibility that may account for the observed pH-dependent, non-enzymatic hydrolysis of GP-2SBPO is via intramolecular cyclization to give cyclo[Gly-Pro] (Scheme 2). Cyclization of dipeptides containing a prolineamide moiety to anhydrides (piperazine-2,5-diones) has been shown previously to occur in aqueous solution.[11] For GP-2SBPO, cyclization may be facilitated by the rigid pyrrolidine ring which helps to position the glycylamine for nucleophilic attack on the adjacent aromatic amide bond[12] (Scheme 2). To corroborate this hypothesis, mass analysis was performed on the GP-2SBPO samples after incubation for 3 h at 37 °C in 0.1 M Tris buffer (pH 7.4) with and without DPP-IV. For the GP-2SBPO sample with DPP-IV, mass peaks corresponding to linear glycylproline and cyclo[Gly-Pro] were observed indicating both enzyme catalyzed hydrolysis and spontaneous cyclization of GP-2SBPO. On the other hand, only a cyclo[Gly-Pro] mass peak was found for the GP-2SBPO sample without DPP-IV indicating spontaneous cyclization of the dipeptide.

To overcome the problem of this unexpected non-enzymatic hydrolysis, addition of a second glycylprolyl sequence to the N-terminus of GP-2SBPO could be an effective solution. Ideally, the extended peptide chain would disfavor intramolecular cyclization, while the initial proteolytic removal of the N-terminal Gly-Pro by DPP-IV would lead to GP-2SBPO, and subsequent liberation of free 2SBPO could be triggered by DPP-IV catalyzed hydrolysis and spontaneous non-enzymatic cyclization (Scheme 3).

As expected, the GPGP-2SBPO was found to be pH stable. Unlike GP-2SBPO, no hydrolysis was observed for GPGP-2SBPO in the absence of DPP-IV between pH 4 and 8 (Fig. 1). The proteolytic hydrolysis of GPGP-2SBPO by DPP-IV was analyzed by fluorescence and absorbance measurements. Under physiological conditions (pH 7.4), no fluorescence signal changes were observed for GPGP-2SBPO alone (Fig. 2). When the probe was incubated with DPP-IV, a steady increase in fluorescence emission was observed giving sixfold increase after 60 min. The fluorescence signal generated at 670 nm indicated catalytic cleavage of the amide bond and release of the fluorescent 2SBPO. Similar to the fluorescence measurements, a sixfold increase in absorbance at 625 nm is observed 60 min after treatment with DPP-IV (Fig. 3). As with GP-2SBPO, the modified GPGP-2SBPO probe is completely water-soluble and requires no additional organic co-solvent for the fluorogenic and chromogenic assays.



Scheme 2. Proposed mechanism for hydrolysis via intramolecular cyclization to release free 2SBPO.



Scheme 3. Proposed schematic representation for the two-step cleavage of GPGP-2SBPO.

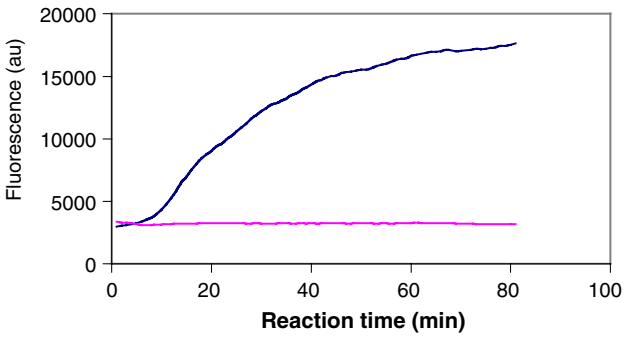


Figure 2. Fluorescence assay for the hydrolysis of GPGP-2SBPO with DPP-IV (18 mU) in 0.1 M HEPES buffer (pH 7.4).

Kinetic parameters for the hydrolysis of GPGP-2SBPO with DPP-IV were further determined under physiological conditions (Table 1). The corresponding parameters for commercially available Gly-Pro-AMC, $(Ala\text{-}Pro)_2$-Rhod110, and Ala-Pro-Rhod110-NH_2[7] are included in the table for comparison. Under the conditions used, the dissociation rate constants (K_m values) indicate that GPGP-2SBPO binds more effectively to DPP-IV than the coumarin-based substrate (Gly-Pro-AMC), but less

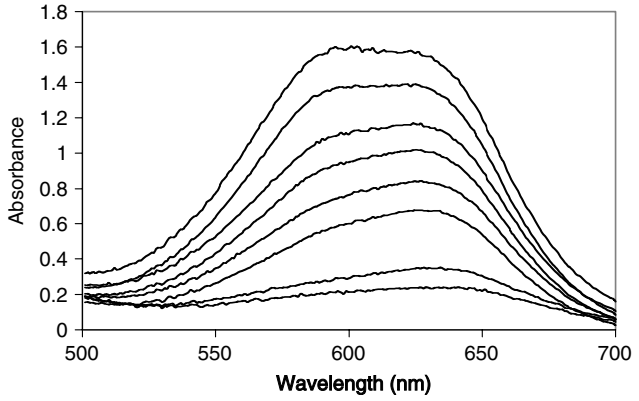


Figure 3. Absorbance assay for the hydrolysis of GPGP-2SBPO with DPP-IV (18 mU) in 0.1 M HEPES buffer (pH 7.4). The curves from bottom to top are at 0, 10, 25, 35, 45, 55, 80, and 95 min, respectively.

Table 1. Kinetic parameters of the fluorogenic substrates for DPP IV

Substrates	K_m (μM)	V_{max} (nM/min)
Gly-Pro-AMC[a]	210.6 ± 3.6	5.2 ± 0.2
$(Ala\text{-}Pro)_2$-Rhod110[a]	15.8 ± 2.4	0.44 ± 0.14
Ala-Pro-Rhod110-NH_2[a]	18.4 ± 1.6	0.32 ± 0.13
Gly-Pro-Gly-Pro-2SBPO	56 ± 8	630 ± 25

[a] Ref. 7.

effectively than the rhodamine-based substrates. A rather large V_{max} value (630 nM/min) for GPGP-2SBPO was obtained. The selectivity constant k_{cat}/K_m value of $2.1 \times 10^3\ M^{-1}\ s^{-1}$ calculated from the kinetic data indicates that the catalytic efficiency of the 2SBPO-based substrate is in the moderate range.

In conclusion, we have synthesized a dual fluorogenic and chromogenic substrate, GPGP-2SBPO, for DPP-IV sensing. The substrate is soluble and stable in aqueous solution without the need for organic co-solvent. Release of water-soluble 2SBPO by DPP-IV can be determined by both fluorometric and colorimetric analysis with long wavelength emission and absorption maxima. A six-fold increase in fluorescence and absorbance was obtained. The kinetic parameters indicate that the catalytic efficiency of DPP-IV to the substrate is high. Potentially, the approach of repeating proteolytic sequence could be applied to design other protease probes. In addition, since this improved DPP-IV substrate emits at far-red region, it could have applications for in vitro high-throughput screening (HTS) and potentially in vivo imaging.

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