

## Photoactivated Coumaryl-diazopyruvate Fluorescent Label for Amine Functional Groups of Tissues Containing Type-I Collagen<sup>¶</sup>

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### ABSTRACT

The design, synthesis and application of a new fluorescent-labeling reagent for collagen has been developed as a prerequisite for the design of a photoactivated collagen-crosslinking compound for surgical wound closure. The amine groups in collagen are the targets of a rational design for a new fluorophore because natural collagen crosslinks are formed between primary (1°) amine groups of lysine and hydroxylysine. The availability of 1° amines for crosslinking in native collagenous tissues was evaluated by reacting tendon and corneal samples with *o*-phthalaldehyde and dansyl chloride, fluorophores commonly used for the detection of 1° and 2° amines. The resulting fluorescent collagen fibrils indicated the presence of amines in native tissue. Subsequently, a photoactivated fluorescent label for 1° and 2° amines, coumaryl gamma-amino-butyric acid diazopyruvate (CGDP), was designed and synthesized. CGDP was first used to photolabel poly-L-lysine, forming a fluorescent, covalent bond to the 1° amine. CGDP was then photoreacted with corneal and tendon tissue samples to produce CGDP fluorescent-labeled samples that were statistically significantly more fluorescent than were the controls. These experiments support the postulate that 1° or 2° (or both) amines in native collagenous tissues are available to serve as targets for photoactivated collagen crosslinkers for wound closure.

### INTRODUCTION

Sutures and staples are the most common means of surgically repairing cut or severed collagenous tissues such as skin, tendons or the cornea. Although sutures and staples provide strong bonds, they

are sometimes difficult to apply (*e.g.* corneal transplantation) and can act as sources of inflammation and infection. The research reported here is aimed at replacing or enhancing sutures using light-activated collagen-crosslinking chemical reagents that bond Type-I collagen, the structural element that provides tissue strength. Type-I collagen is the major fibrillar collagen in the dermis (1,2), tendons (3) and corneal stroma (4,5), tissues that are 70–80% collagen by dry weight. To design and synthesize photochemical collagen-crosslinking reagents, it is essential to determine the potential bonding sites in native tissues rich in Type-I collagen.

Type-I collagen protein consists of three  $\alpha$ -helical amino acid chains that together form a right-handed triple helix. Every third amino acid residue in the  $\alpha$  chain is glycine (Gly). The two intervening amino acids can vary, but proline (Pro) occurs frequently, whereas lysine (Lys) and hydroxylysine (Hyl) are much less frequent (6). Intermolecular crosslinks are essential for the formation of collagen fibrils, fibers and tissues, and play a major role in determining the net strength of the tissue (7). Intermolecular crosslinks can be formed by both enzymatic and nonenzymatic mechanisms but are only found between Lys or Hyl amino acid residues in the nonhelical C and N terminal ends of the protein and the adjacent helical domain (8–10).

Because naturally occurring collagen crosslinks are formed from Lys and Hyl amino acids, we believe that artificial, photochemical collagen crosslinking would most likely be achieved by targeting free amine groups on those amino acids. There are, however, a limited number of such potential crosslinking sites per collagen protein, and those sites can become naturally crosslinked through various chemical processes with aging. Consequently, there may not be sufficient numbers of unoccupied Lys and Hyl sites available in collagenous tissues for photochemical crosslinking. Further, tissues that contain predominantly Type-I collagen also contain other proteins, including other collagen types, that may have free primary (1°) and secondary (2°) amines. Thus, it is important first to determine the extent to which free 1° and 2° amines are present in native collagenous tissue.

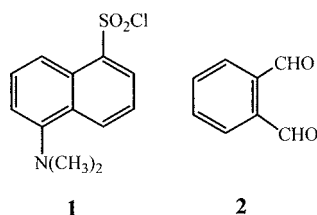
In this report we describe (1) fluorescent-labeling experiments that demonstrate the availability of free 1° amine groups in tissues containing Type-I collagen; and (2) the design, synthesis and testing of a novel photoactivated fluorophore that bonds covalently to free 1° amine groups. For fluorescent labeling, two fluorophores were used: dansyl chloride (1), which is specific for both 1° and 2° amines (11–13), and *o*-phthalaldehyde (2), which is specific for 1° amines

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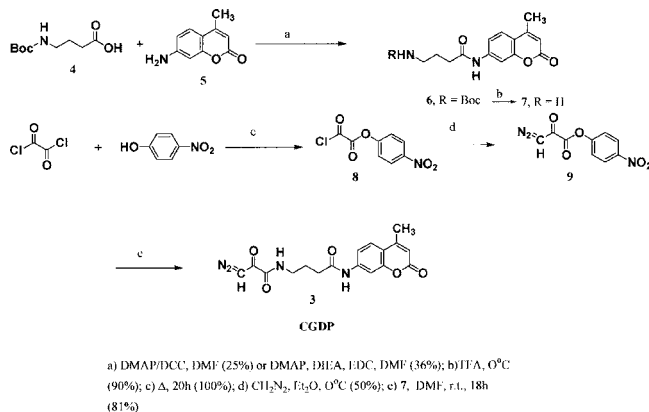
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**Abbreviations:** CGDP, coumaryl gamma-amino-butyric-acid diazopyruvate; DMAP, 4-(dimethylamino)pyridine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; EA, ethyl acetate; FAB-MS, fast atom bombardment-mass spectroscopy; GABA, gamma-amino-butyric acid; Gly, glycine; Hyl, hydroxylysine; Lys, lysine; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; Pro, proline; RGB, red-green-blue; ROI, region of interest.

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(14–16). Our photoactivated fluorophore (coumaryl gamma-aminobutyric acid diazopyruvate [CGDP] [3, Scheme 1]) was tested with solutions of poly-L-lysine and with tissue samples.



Scheme 1.

## MATERIALS AND METHODS

### Dansyl chloride and *o*-phthalaldehyde tissue fluorescent labeling

Achilles tendons from 3–6 month old white male New Zealand rabbits were collected and stored at  $-70^{\circ}\text{C}$  using a previously described procedure (17). For experiments, frozen tendons were thawed, cleaned of fascia and cut lengthwise into pieces of approximately  $1 \times 1 \times 7$  mm. Tendon samples were then immersed in solutions of (1) 0.1 *M* dansyl chloride (1) in acetone; (2) 0.1 *M* *o*-phthalaldehyde (2) in acetone; or (3) acetone alone (control), for 24 h in the dark at  $4^{\circ}\text{C}$ . Subsequently, tendon samples were extensively washed in three changes of acetone over 36 h. For microscopy, tendon samples were briefly rinsed in 0.9% saline and manually disrupted using forceps. Tissue fluorescence was examined with an Olympus Fluoview 300 confocal scanning laser microscope with 488 nm excitation light. Fluorescent and transmitted light images were captured using the microscope's software. Excitation light intensity, photomultiplier tube voltage, gain and offset were identical for all images. Twenty repeated scans were averaged to improve the signal-to-noise ratio in each recorded image.

### Synthesis of photoactivated fluorescent labels for 1° and 2° amines (see Scheme 1)

*N*-(4-methylcoumar-7-yl)-*N*<sup>t</sup>-*t*-Boc- $\gamma$ -aminobutyramide. According to the method of Lee and Lown (18), a mixture of 0.990 g (5.14 mmol) of 7-amino-4-methylcoumarine (5), *N*-*t*-Boc- $\gamma$ -aminobutyric acid (4, 1.64 g, 8.28 mmol) and 4-(dimethylamino)pyridine (DMAP; 95.0 mg, 0.770 mmol) was dissolved in dry dimethylformamide (DMF) (18 mL) and then chilled ( $0^{\circ}\text{C}$ ). A solution of dicyclohexylcarbodiimide (1.76 g, 8.54 mmol) in 10 mL dry DMF was introduced. The reaction mixture was stirred under an argon atmosphere at  $0^{\circ}\text{C}$  for 15 min and at room temperature for 19 h, after which thin-layer chromatography (silica gel, Et<sub>2</sub>O–MeOH 11:1) indicated that the reaction was complete. The precipitated urea was removed by filtration, and the filtrate was concentrated under high vacuum to give a yellow residue. The residue was purified by silica gel column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/ethyl acetate [EA] 7:3). After collection of

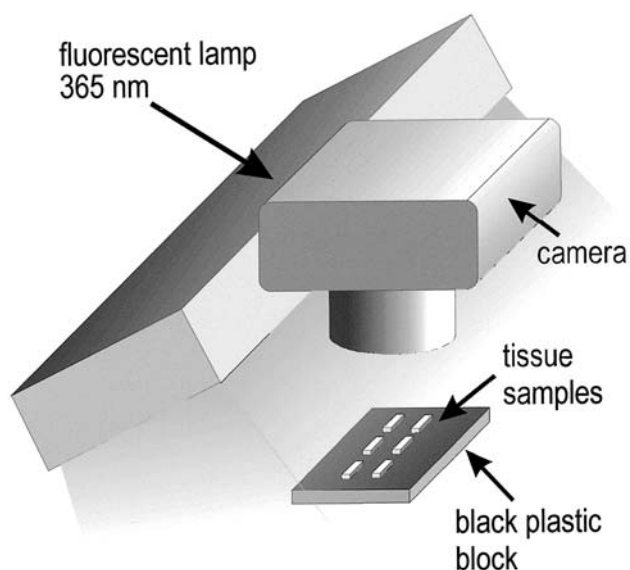
appropriate fractions containing the product, the solvent was evaporated and gave light-yellow crystals of *N*-(4-methylcoumar-7-yl)-*N*<sup>t</sup>-*t*-Boc- $\gamma$ -aminobutyramide (6) (458 mg, 1.21 mmol, 25%); mp  $190^{\circ}\text{C}$ ; IR (KBr) 3368, 2979, 2930, 1691, 1619, 1580, 1524, 1393, 1367, 1336, 1269, 1251, 1169, 873, 849  $\text{cm}^{-1}$ ; <sup>1</sup>H-nuclear magnetic resonance (NMR) (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 9.67 (br, 1H), 7.75 (dd, 1H, *J* = 8.80 Hz, *J* = 1.20 Hz), 7.66 (d, 1H, *J* = 1.20 Hz), 7.52 (d, 1H, *J* = 8.80 Hz), 6.18 (s, 1H), 4.88 (br, 1H), 3.25 (m, 2H), 2.42 (m, 2H), 2.40 (s, 3H), 1.87 (m, 2H), 1.47 (s, 9H); <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  = 171.8, 161.4, 157.6, 154.2, 152.4, 142.2, 125.1, 115.8, 115.7, 113.1, 106.9, 80.3, 39.1, 34.6, 28.4, 27.7, 18.6; fast atom bombardment–mass spectroscopy (FAB-MS) *m/z* (relative intensity) 361 (*M* + 1, 25); exact mass calcd for C<sub>19</sub>H<sub>25</sub>O<sub>5</sub>N<sub>2</sub>; 361.1763, found: 361.1779.

*Alternative synthesis of N*-(4-methylcoumar-7-yl)-*N*<sup>t</sup>-*t*-Boc- $\gamma$ -aminobutyramide (6). According to the method of Desai *et al.* (19), a mixture of 0.900 g (5.14 mmol) 7-amino-4-methylcoumarine (5), *N*-*t*-Boc- $\gamma$ -aminobutyric acid (4, 1.04 g, 5.14 mmol) and DMAP (63.0 mg, 0.510 mmol) was dissolved in dry DMF (14 mL) and then chilled ( $0^{\circ}\text{C}$ ). A solution of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (2.96 g, 15.4 mmol) and diisopropylethylamine (1.99 g, 15.4 mmol) in 15 mL dry DMF was introduced. The reaction mixture was stirred under an argon atmosphere at  $0^{\circ}\text{C}$  for 15 min and at room temperature for 48 h, at which time thin-layer chromatography (silica gel, Et<sub>2</sub>O–MeOH 11:1) indicated that the reaction was complete. The reaction solution was concentrated under high vacuum to give a green oily residue. The oil was added to 200 mL of ice water with stirring. The precipitate was collected by filtration and then purified by column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/EA 7:3). After collection of the appropriate fractions for the product, the solvent was evaporated and gave light yellow crystals of *N*-(4-methylcoumar-7-yl)-*N*<sup>t</sup>-*t*-Boc- $\gamma$ -aminobutyramide (6) (0.666 g, 1.71 mmol, 36%); mp  $190^{\circ}\text{C}$ ; IR, <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, FAB-MS and exact mass were identical to those reported above.

*N*-(4-methylcoumar-7-yl)- $\gamma$ -aminobutyramide (7). A solution of *N*-(4-methylcoumar-7-yl)-*N*<sup>t</sup>-*t*-Boc- $\gamma$ -aminobutyramide (6) (0.666 g, 1.85 mmol) in 12 mL trifluoroacetic acid was cooled to  $0^{\circ}\text{C}$  and stirred for 4 h. The resulting solution was concentrated by a rotary evaporator and the solvent removed under vacuum. The crude product was dissolved in H<sub>2</sub>O and extracted with EA. The aqueous layer was collected, and the water was lyophilized to give white crystals of *N*-(4-methylcoumar-7-yl)- $\gamma$ -aminobutyramide trifluoroacetate salt (7) (0.632 g, 1.67 mmol, 90%); mp  $197^{\circ}\text{C}$ ; IR (KBr) 3479, 3437, 3297, 3066, 1696, 1670, 1620, 1583, 1530, 1417, 1400, 1205, 1184, 1158  $\text{cm}^{-1}$ ; UV–vis (MeOH)  $\lambda_{\text{max}}$ ( $\epsilon$ ) 327(20479), 296(13572), 228(23879), 210(39823); <sup>1</sup>H-NMR (400 MHz, D<sub>2</sub>O)  $\delta$  = 7.58 (d, 1H, *J* = 8.8 Hz), 7.49 (s, 1H), 7.24 (d, 1H, *J* = 8.8 Hz), 3.03 (m, 2H), 2.53 (m, 2H), 2.34 (s, 3H), 1.98 (m, 2H); <sup>13</sup>C-NMR (100 MHz, D<sub>2</sub>O)  $\delta$  = 173.9, 164.8, 156.4, 153.5, 141.1, 126.3, 117.2, 116.8, 112.4, 107.9, 39.2, 33.6, 22.9, 18.2; FAB-MS *m/z* (relative intensity) 261 (*M* + 1, 67); exact mass calcd for C<sub>14</sub>H<sub>17</sub>N<sub>2</sub>O<sub>3</sub>; 261.1239, found: 261.1231.

*p*-Nitrophenyl oxalyl chloride (8) and *p*-nitrophenyl 3-diazopyruvate (9). These were prepared according to the method of Goodfellow *et al.* (20) and Andrt (21).

*Synthesis of photoactivated fluorophore N*-(4-methylcoumar-7-yl)-*N*<sup>t</sup>-(3'-diazopyruvoyl)  $\gamma$ -aminobutyramide (CGDP, 3). According to the method of Goodfellow *et al.* (20), *N*-(4-methylcoumar-7-yl)- $\gamma$ -aminobutyramide trifluoroacetate salt (7), (100 mg, 0.270 mmol) was dissolved in 0.5 mL dry DMF in oven-dried glassware under argon, to which *p*-nitrophenyl-3-diazopyruvate (9, 63.0 mg, 0.270 mmol) was added with vigorous stirring. The reaction was stirred for 18 h under an atmosphere of argon, in a flask protected from light by foil. Thin-layer chromatography (silica gel, Et<sub>2</sub>O–MeOH 11:1) indicated that the reaction was complete. Diethyl ether (12 mL) was added to the reaction, and the resulting light brown suspension was taken up in a Pasteur pipette and transferred to a 12 mL centrifuge tube. After intensive shaking and 5 min of centrifuging, the ether was decanted, and the crude product was dispersed thoroughly with a glass rod in 12 mL of fresh ether and spun. This process was repeated six times. The crude product was dried under high vacuum. Further purification by flash column chromatography (MeOH) gave light yellow crystals of CGDP (3). The elution of the product should be carried out as quickly as possible to avoid decomposition of the products on the column. The yield was 77.0 mg, 0.216 mmol, 81%; mp  $183^{\circ}\text{C}$ , dec.; IR (KBr) 3433, 3331, 3098, 2925, 2853, 2143, 2107, 1686, 1618, 1586, 1528, 1378  $\text{cm}^{-1}$ ; UV–vis (MeOH)  $\lambda_{\text{max}}$ ( $\epsilon$ ) 324.5(20251), 316(20589), 299(17469), 229(23991), 210(37550); fluorescence (MeOH)  $\lambda_{\text{em}}$  ( $\lambda_{\text{ex}}$ ) 385 nm (340 nm); <sup>1</sup>H-NMR (400 MHz, dimethylsulfoxide [DMSO]-*d*<sub>6</sub>)  $\delta$  = 10.34 (s, 1H), 8.76 (m, 1H), 7.74 (d, 1H, *J* = 1.8 Hz), 7.69 (d, 1H, *J* = 8.7 Hz), 7.45 (dd, 1H, *J* =



**Figure 1.** Arrangement for photographically recording tendon or corneal tissue fluorescence.

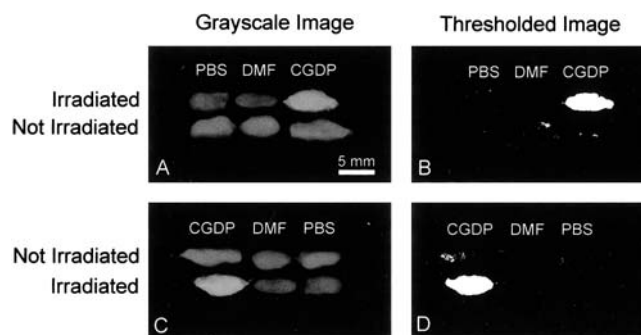
8.7 Hz,  $J = 1.8$  Hz), 6.59 (s, 0.83H), 6.24 (s, 1H), 5.82 (s, 0.17H), 3.18 (m, 2H), 2.38 (s, 3H), 2.35 (m, 2H), 1.79 (m, 2H);  $^{13}\text{C}$ -NMR (125 MHz,  $\text{DMSO-d}_6$ )  $\delta = 182.0, 171.6, 160.3, 160.1, 153.7, 153.2, 142.6, 125.9, 115.1, 114.8, 112.1, 105.5, 55.2, 38.4, 33.8, 24.4, 18.0$ ; MS ( $m/z$ ) 356 ( $\text{M}^+$ , 3.1%), 286 ( $\text{M}-\text{COCHN}_2$ , 12.5%), 271 (55.0%), 243 ( $\text{M}-\text{NHCOCOCHN}_2$ , 83%), 175 (44.6%), 147 (35.3%), 85 (23.9%), 69 (89.2%).

#### Photoactivated CGDP tissue labeling

Both the rabbit Achilles tendon and corneal samples were tested. Achilles tendon samples were prepared as described above. Corneas were dissected from rabbit eyes 1 h or less post mortem. Corneal epithelial and endothelial cell layers were removed by scraping with a scalpel. Both corneal and tendon samples were cut into strips of approximately  $1 \times 1 \times 7$  mm. Two tissue pieces were placed in each of three quartz cuvettes containing (1) phosphate-buffered saline (PBS)—control 1; (2) DMF alone—control 2; and (3) a 20 mM solution of CGDP in DMF. The tissue pieces were soaked in the various solutions in the dark for 1 h and were then removed for irradiation. An identical set of tissue in cuvettes remained in the dark. The three cuvettes containing tissue for irradiation were placed in a slowly rotating merry-go-round apparatus in a Rayonet Photoreactor (Southern New England Co., Branford, CT) that consisted of 12 fluorescent Rayonet RPR 350 nm lamps. After 40 min of exposure the tissue samples were removed from the cuvettes and washed three times for 20 min with gentle stirring in PBS or DMF to remove any unreacted CGDP. Finally, the tissue samples were washed two times in PBS to remove any residual DMF. The set of dark-control tissue samples (nonirradiated control) were washed in an identical manner.

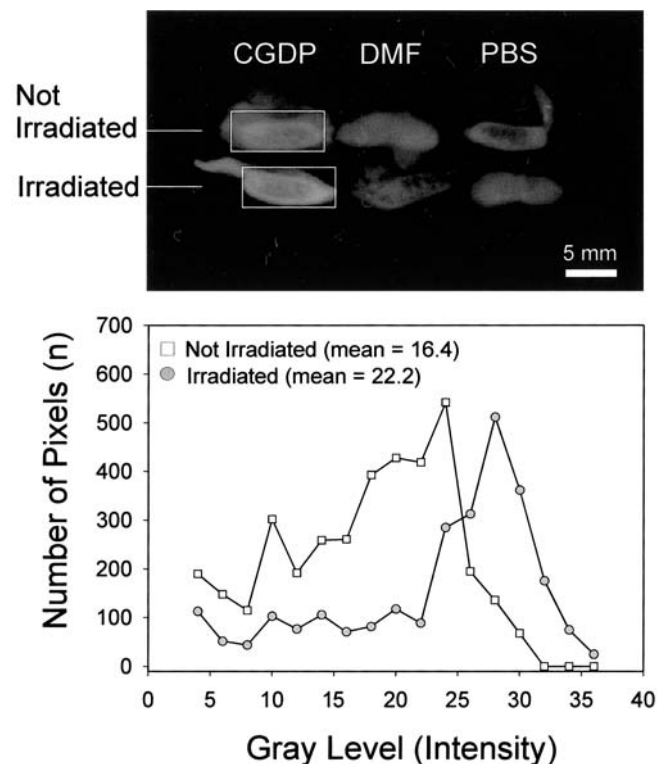
One tissue sample from each cuvette (PBS, DMF, DMF + CGDP irradiated and nonirradiated) was placed on a black plastic background beneath a fluorescent UV excitation lamp ( $\lambda_{\text{peak}} = 365$  nm) (Fig. 1). The lamp was  $\sim 30$  cm above the tissue and tilted at an angle of  $\sim 10^\circ$  to the vertical. A camera fitted with a macrolens and Kodak Elite Chrome film was placed so that it was directly above the tissue block. Because the fluorescent lamp was tilted, the tissue in the row nearer the lamp was conceivably illuminated more brightly than that in the row farthest from the lamp. To control for this possibility, additional photographs of tissue fluorescence were made with the block rotated  $180^\circ$  so that the tissue that first had received less light during photography now received more. A graded series of camera exposures were made with the tissue block in both positions to ensure that the recorded tissue fluorescence was adequately high but did not saturate the exposure. Selected slides from the exposed film were digitized as red-green-blue (RGB) images using a slide digitizer.

The Red and Green components of the digitized images were eliminated

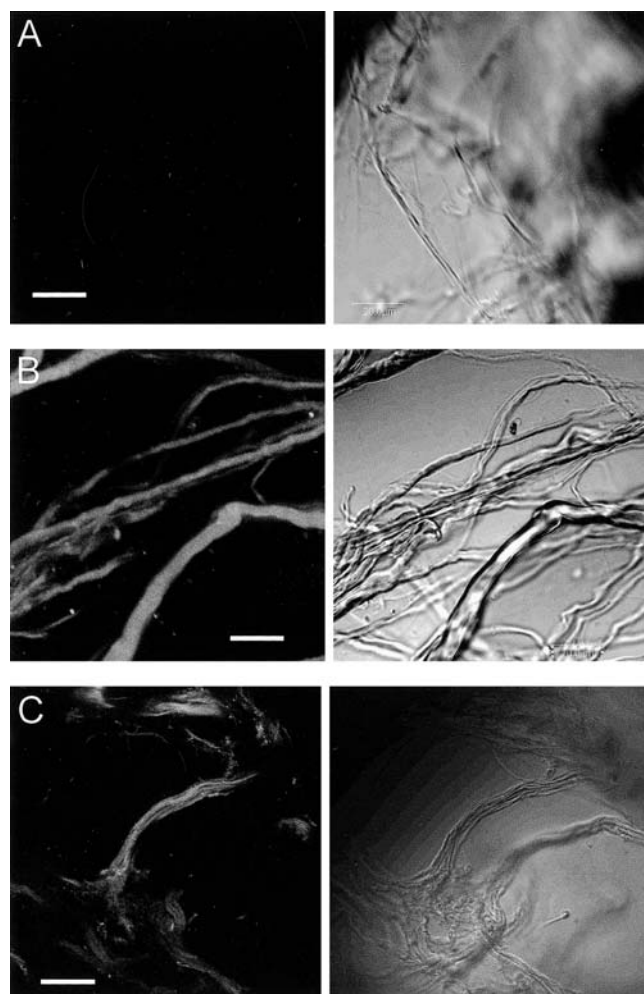


**Figure 2.** Digitized fluorescence images of rabbit Achilles tendon samples. A: Grayscale (monochrome) digitized image of the Blue component of the original RGB photographic image. B: Thresholded version of (A) showing the greatest fluorescence of irradiated CGDP tissue. C: Same as (A) except that the block holding tissue was rotated  $180^\circ$  with respect to the excitation lamp. D: Thresholded image of (C) showing the greatest fluorescence for the irradiated CGDP tissue sample. CGDP, tissue treated with CGDP in DMF; DMF, tissue treated with DMF only; PBS, tissue treated with PBS only.

because it was found that there was no fluorescence in these spectral ranges. The remaining Blue image was converted to an 8-bit grayscale image as shown in Fig. 2. Two types of analysis were performed on the grayscale images. First, the image was simply thresholded to gain a rapid assessment of tissue fluorescence. With this technique all pixels below a certain level (the threshold) are turned to black (*i.e.* level 0 on the 256-level, 8-bit scale), whereas all the pixels above the threshold are turned to white (*i.e.* level 256). In this way the darker tissue samples (or portions of the samples) disappear, and only the brightest are left, indicating the most fluorescent



**Figure 3.** Grayscale digital image of the Blue component of the RGB image of rabbit Achilles tendon samples. Rectangles show ROI for which pixel grayscale values (intensities) were recorded (top) and histograms of grayscale (intensity) values for the two ROI shown in the top panel (bottom).



**Figure 4.** Confocal scanning laser microscope images of rabbit Achilles tendon collagen fibrils. Left image in each panel is of fluorescence; right image is of transmitted light. A: Control tendon sample treated with acetone alone. B: Tendon sample treated with *o*-phthalaldehyde. C: Tendon sample treated with dansyl chloride. White calibration bar represents 20  $\mu\text{m}$ .

tissue. Figure 2 demonstrates that after thresholding, the only tissue samples that are visible are those reacted with CGDP and only the irradiated sample is entirely visible. This means that the tissue sample that was reacted with CGDP and irradiated had the highest residual fluorescence from the coumaryl group. Rotating the tissue samples 180° (Fig. 2C,D) resulted in a pattern of fluorescence very similar to that of the nonrotated samples in this and in subsequent measurements.

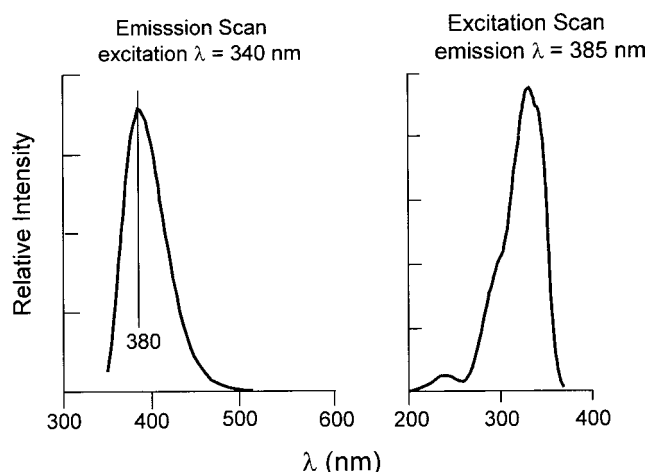
The fluorescent intensity of each tissue sample was measured by enclosing the digitized image of the sample within a region of interest (ROI) rectangle using an image analysis program (Matrox Inspector, Matrox Electronic Systems Ltd., Dorval, Québec, Canada) (Fig. 3). The gray level of each pixel within the ROI was recorded and used to plot a gray-level histogram and to calculate a mean gray-level intensity. Mean gray-level intensity,  $\bar{I}$ , was calculated as

$$\bar{I} = \frac{\sum_{i=0}^N n_i L_i}{N}$$

where  $n_i$  is the number of pixels of gray-scale value  $L_i$ , and  $N$  is the total number of pixels in the ROI.

#### Light-activated bonding of CGDP to poly-L-lysine

A 10 mM solution of CGDP in 50  $\mu\text{L}$  DMSO was prepared, to which was added 250  $\mu\text{L}$  of 0.1 M NaOH solution. A  $\sim 5$  mM solution of poly-L-



**Figure 5.** Excitation and emission spectra of CGDP.

lysine hydrobromide (Sigma Chemical Co., St. Louis, MO; mean molecular weight, 22 kDa) in 1.2 mL of 0.1 M NaOH was prepared and added to the CGDP solution to ensure the presence of free amino groups of poly-L-lysine. The CGDP + poly-L-lysine solution was divided into three equal aliquots of 0.5 mL. One aliquot was irradiated for 40 min as described previously. The two other aliquots served as controls and were not irradiated. The irradiated sample and one control sample were placed separately in dialysis tubing (10 kDa cutoff) and immersed in 500 mL of pH 7.4 PBS to remove the unreacted CGDP. Dialysis was carried out in the dark at 4°C, with gentle stirring of the buffer. The dialysis buffer was changed four times during the 44 h dialysis. The other control sample was placed in a sealed vial, wrapped in foil and maintained in the dark at 4°C.

After dialysis, samples were placed in sealed 1.0 mL quartz cuvettes and placed on a horizontally oriented 365 nm UV excitation lamp (*i.e.* the excitation light was directed upward from beneath the cuvette). The array of cuvettes was photographed in color, and selected slides were digitized as RGB images as described previously.

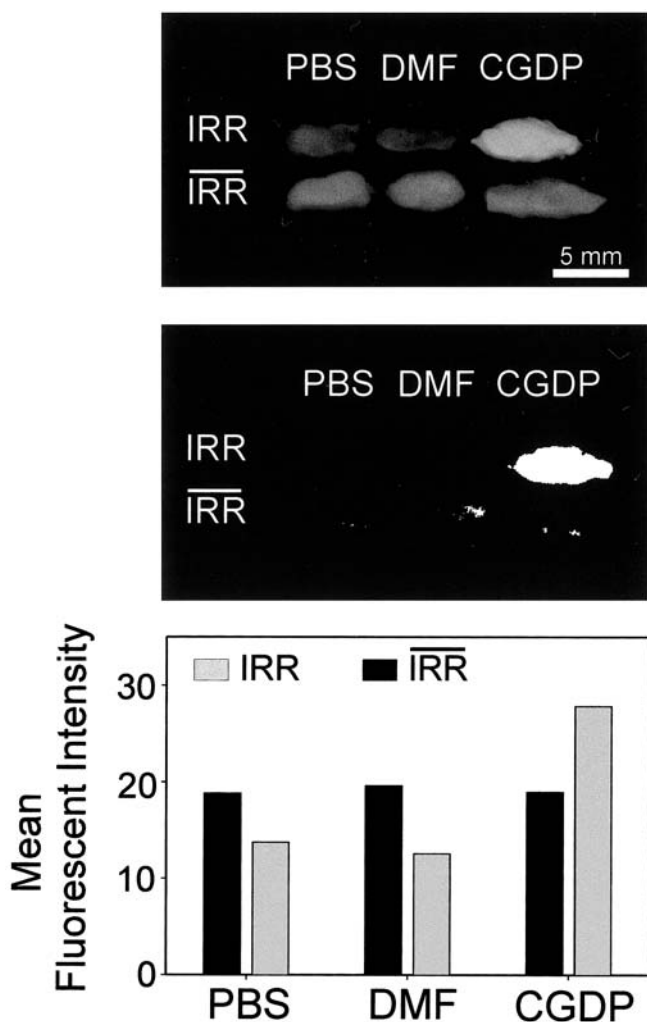
## RESULTS

### Dansyl chloride and *o*-phthalaldehyde fluorescent labeling

Transmitted and fluorescence images of tendon fibrils treated with acetone (control), *o*-phthalaldehyde (2) and dansyl chloride (1) are shown in Fig. 4. Control samples treated with acetone alone had extremely low levels of fluorescence, resulting in a black fluorescence image (Fig. 4A). Tendon collagen fibrils treated with *o*-phthalaldehyde (2) (Fig. 4B) and dansyl chloride (1) (Fig. 4C) fluoresced intensely, indicating that these compounds were chemically bound to 1° and perhaps to 2° amine groups.

### Synthesis of photoactivated fluorescent labels for 1° and 2° amines

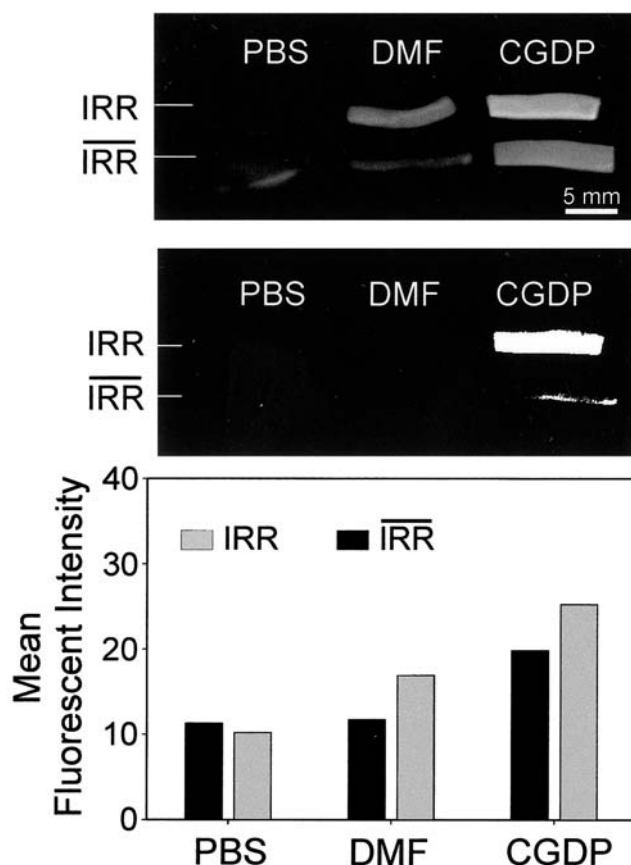
The three sections of CGDP (3, Scheme 1) are a fluorescent coumarin moiety connected through a gamma-amino-butyric acid (GABA) spacer to the photoactive diazopyruvate. A six-step, convergent synthesis of CGDP (3) is shown in Scheme 1. The diazopyruvyl functional group was introduced on the coumaryl-GABA tether by displacement of *p*-nitrophenol from the *p*-nitrophenyl diazopyruvate (9). CGDP (MW 340) is a yellow-white powder, soluble in DMF, DMSO and other polar organic solvents. In solution, unreacted CGDP fluoresces with a peak wavelength of 380 nm and is maximally excited at  $\lambda_{\text{excitation}} = 330$  nm (Fig. 5).



**Figure 6.** Fluorescence measurements of treated rabbit Achilles tendon samples. Grayscale image of the Blue component of digitized photograph (top), thresholded image of top image (middle) and mean grayscale intensity of each sample (bottom). IRR, irradiated samples; IRR, non-irradiated; PBS, phosphate-buffered saline; DMF, dimethylformamide; CGDP: coumaryl-GABA-diazopyruvate in DMF.

#### Photoactivated CGDP tissue labeling

Grayscale and thresholded images of rabbit Achilles tendon samples are shown in Fig. 6. Even in the grayscale image it is apparent that the irradiated sample containing CGDP is the most fluorescent. This difference is more pronounced in the thresholded images, where only the irradiated CGDP-treated tissue samples are fully visible. Measurements of mean fluorescent intensity (Fig. 6, bottom panel) indicated that the irradiated CGDP-treated tissue was more fluorescent than all the other samples. Nonirradiated tissue fluorescent intensities for all treatments (PBS, DMF, CGDP) were approximately equal (18–20 units) and probably resulted from the intrinsic fluorescence of collagenous tissue. This result also implies that the washing procedure removed any unbound CGDP from the tissue because nonirradiated CGDP-treated tissue was no more fluorescent than the tissue treated with PBS or DMF alone. Tissue irradiated in the presence of PBS or DMF only is less fluorescent than the nonirradiated tissue. Only when the tissue was treated with CGDP and irradiated did tissue fluorescence increase.



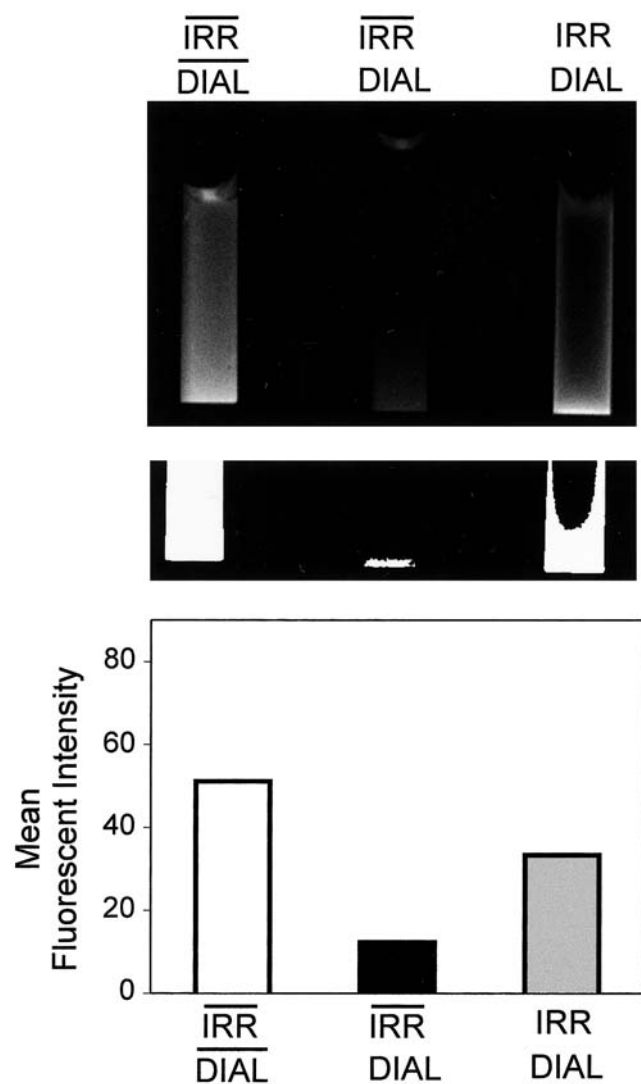
**Figure 7.** Fluorescence measurements of treated rabbit corneal stromal samples. Grayscale image of the Blue component of digitized photograph (top), thresholded image of top image (middle) and mean grayscale intensity of each sample (bottom). IRR, irradiated samples; IRR, non-irradiated; PBS, phosphate-buffered saline; DMF, dimethylformamide; CGDP, coumaryl-GABA-diazopyruvate in DMF.

Figure 7 is an example of the same measurements described above, but with rabbit corneal tissue. Again, it is clear that the most fluorescent tissue sample was the one treated with CGDP and irradiated.

The tissue irradiation experiments described above were repeated eight times, twice with corneal tissue and six times with tendon. Mean fluorescent intensity of the pooled irradiated tissue samples ( $23.6 \pm 4.6$  [mean  $\pm$  SD]) was statistically significantly greater than that of the pooled nonirradiated samples ( $19.5 \pm 4.0$ ) ( $P \leq 0.005$ : paired *t*-test,  $t = 3.98$ ,  $df = 7$ ). Mean fluorescent intensity of the irradiated samples was also statistically significantly greater than that of the nonirradiated samples ( $24.6 \pm 2.6$  and  $19.7 \pm 2.5$ , respectively) when the block that held the tissue samples beneath the excitation lamp was rotated  $180^\circ$  ( $P \leq 0.001$ : paired *t*-test,  $t = 7.00$ ,  $df = 7$ ), implying that the tissue samples were evenly illuminated.

#### Photoactivated bonding of CGDP to poly-L-lysine

Photochemical attachment of CGDP to poly-L-lysine is illustrated in Fig. 8, which shows fluorescence images of the contents of the three cuvettes. The nonirradiated, nondialyzed solution is brightest because it contained the entire amount of unreacted CGDP (Fig. 8, top left). The irradiated, dialyzed solution (Fig. 8, top right) is brighter than the nonirradiated, dialyzed solution, indicating that



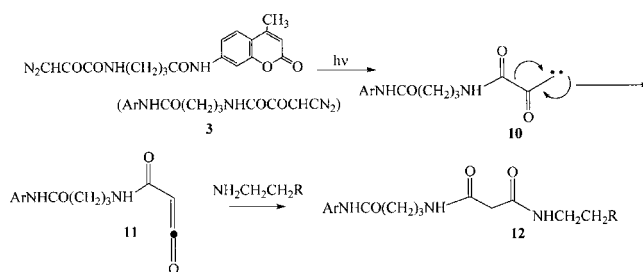
**Figure 8.** Digitized grayscale image of the Blue component of the RGB photograph of poly-L-lysine + CGDP in quartz cuvettes resting on a UV excitation lamp (top), thresholded image of lower ~1 cm of fluorescence from cuvettes (middle) and mean grayscale intensity of bottom 1 cm of each cuvette (bottom). DIAL, dialyzed; IRR, irradiated;  $\overline{\text{IRR}}$ , nonirradiated;  $\overline{\text{DIAL}}$ , nondialyzed.

CGDP was bound to the poly-L-lysine. Intensity measurements of the fluorescence were made from equal areas extending from the bottom of each cuvette upward. The results of these measurements (Fig. 8, bottom panel) are consistent with the visual observations. Thus, it is reasonable to assume that CGDP has been photochemically bonded to the amine groups of poly-L-lysine. Fluorescent intensity of irradiated, dialyzed CGDP + poly-L-lysine was approximately 76% that of the nonirradiated, nondialyzed solution, indicating that a substantial proportion of CGDP was bound to poly-L-lysine.

## DISCUSSION

### Reactive amine groups are present in collagenous tissues rich in Type-I collagen

The fluorescent-labeling experiments with dansyl chloride described above indicate that 1° or 2° (or both) amine sites are present



**Scheme 2.**

in the tendon as shown previously by Vidal (12). Labeling experiments with *o*-phthalaldehyde demonstrate that 1° amine sites are available for bonding in tendon samples rich in Type-I collagen. It is reasonable to assume that the amine groups are the Lys and Hyl amino acids of Type-I collagen because Type-I collagen is the most prevalent protein in tendon tissue. However, the possibility that dansyl chloride and *o*-phthalaldehyde were bound to amine groups in the interfibrillar matrix cannot be ruled out. The matrix surrounding the collagen fibrils contains other molecular components that might bond the fluorophores. In the corneal stroma these include other types of collagen (V, VI) as well as proteoglycans containing keratin sulfate or dermatin sulfate glycosaminoglycan (GAG) chains and specialized fibroblast cells (keratocytes) (22). The tendon contains similar molecular components (1).

### Photoactivated CGDP reacts with amines

In probing for active amine sites, a collagen sample was exposed to a solution of CGDP (3) and irradiated with near-UV or visible (blue) light (Scheme 2). Photolysis of CGDP (3) generates a highly reactive ketoketene intermediate 11 that itself is formed by a Wolff rearrangement ( $3^* \rightarrow 11$ ). The rearrangement is believed to involve the intermediacy of carbene 10 (23). In fact, the coumaryl-GABA-ketoketene (11) is a rationally designed reactive intermediate that reacts selectively with free 1° and 2° amine groups in collagen to form a fluorescently labeled amine bis malonamide (12). The resultant fluorescence from the thoroughly washed labeled collagen provides a direct measure of the extent of covalent attachment of the tethered CGDP to the available amine sites on collagen.

### Photoactivated CGDP bonds to collagenous tissue and amine groups of Lys

It is likely that CGDP formed covalent bonds with 1° amines of collagen. However, as with dansyl chloride and *o*-phthalaldehyde, we cannot rule out the possibility that other proteins with 1° or 2° amines in the tendon and corneal samples were involved in bonding. The demonstration that CGDP produces a highly fluorescent product when irradiated with poly-L-lysine shows that amines on Lys are sufficient for CGDP labeling. The active ketene of photon-activated diazopyruvate can, however, interact with other nucleophiles; thus, it is conceivable that CGDP was bound to functional groups other than an amine.

In summary, we have presented evidence that reactive 1° amine groups are present in native collagenous tissues and that our synthetic, photoactivated fluorescent probe (CGDP) bonds to these groups. This study has opened the way for the design and synthesis of

bi- and poly-functional diazopyruvates as crosslinking agents for collagenous tissues, thereby acting as photostitches for wound repair.

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