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Synthesis of Immunoreagents for Detection of Deoxypyrrololine, a Cross-link of Bone Collagen

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Abstract—Two immunogens (**5**,**6**) and two probes (fluorescent **7** and chemiluminescent **8**) were prepared from benzyl ester (−)-**10**. These immunoreagents (**5**,**6** and **7**,**8**) are useful for detection of collagen cross-link (+)-deoxypyrrololine (Dpl, **4**), and for development of assays for osteoporosis. © 2000 Elsevier Science Ltd. All rights reserved.

Bone is composed of 90% of collagen, a family of proteins, which governs its biochemical properties and integrity.[1] Lysine and hydroxylysine constitute two important building blocks of bone collagen. During the process of bone maturation, the lysine and hydroxylysine residues undergo enzymatic transformations, catalyzed by lysyl oxidase, to form the cross-links between the neighboring collagen molecules. Two types of cross-links, pyridinium cross-links (pyridinoline (Pyd, **1**), deoxypyridinoline (Dpd, **2**)) (Fig. 1) and pyrrole cross-links (pyrrololine (Pyl, **3**), deoxypyrrololine (Dpl, **4**)) are formed in this enzymatic process.[2,3] Bone formation and resorption, the two important processes of bone metabolism, can be assessed by studying the bone matrix components, e.g. cross-links (**1**–**4**). Additionally, in recent years, the pyridinium cross-links (**1**,**2**) have attracted much attention as clinical markers for osteoporosis.[4] However, the studies of pyrrole cross-links (**3**,**4**) were hampered due to its nonavailabity and unstableness to the isolation conditions which require acidic hydrolysis.[5–7] In this context, we have recently synthesized the pyrrole cross-link, (+)-Dpl (**4**)[8] to facilitate the studies on pyrrole cross-links. In this paper, we report the synthesis of immunoreagents (immunogens (**5**,**6**) and probes (**7**,**8**)), which are needed for detection of (+)-Dpl (**4**), and for studies of bone metabolism by immunoassay techniques (for studies on pyridinium cross-links, see refs 9–11).[12,13]

Since the low molecular weight compounds (e.g. cross-links **1**–**4**) do not as such induce the formation of antibodies, they need to be conjugated to a carrier protein, thus forming an immunogen.[14,15] These immunogens are then used for inoculation of an animal (e.g. mice, hamster, rabbit, sheep) for production of antibodies. There are a number of important issues which need to be considered when preparing the immunogens, such as: site of the analyte modification, selection of carrier protein, choice of linking arm, method of conjugation and the hapten density.[16,17] More importantly, the chirality of optically active analytes (e.g. Dpl, **4**), needs to be preserved in preparing the immunoreagents. In order to allow the host animal's immune system to recognize the chiral centers and overall structural topology of Dpl (**4**), we envisioned site specific attachment at the 2-position via amide functionality with incorporation of 6-aminohexanoic acid as a spacer between Dpl (**4**) and carrier protein. This is important, since in the case of thyroxine (T_4),[18,19] we observed that the *anti*-thyroxine antibodies, which were raised from an immunogen prepared by direct conjugation of the carboxylic acid of T_4 to carrier protein, were blind to the chiral center. Additionally, since the pyrrole cross-links are reported to be unstable, the amide functionality at 2-position of pyrrole ring in the immunoreagents will increase their stability. Bovine serum albumin (BSA, molecular weight: 66,430 Da) and keyhole limphet hemocyanin (KLH, molecular weight: 450–13,000 kDa) are the two commonly used carrier proteins for the preparation of immunogens. Although the immunogens prepared using KLH as carrier protein are difficult to characterize due to the poor solubility and variable mass, they are frequently used for production of antibodies. Since the antibodies produced for carrier protein are also present in the host animal bleeds, an

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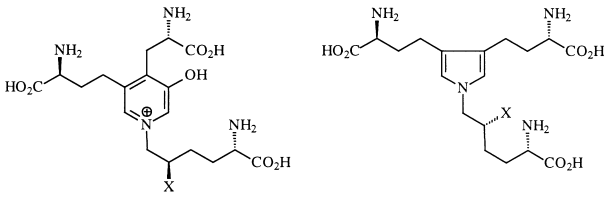


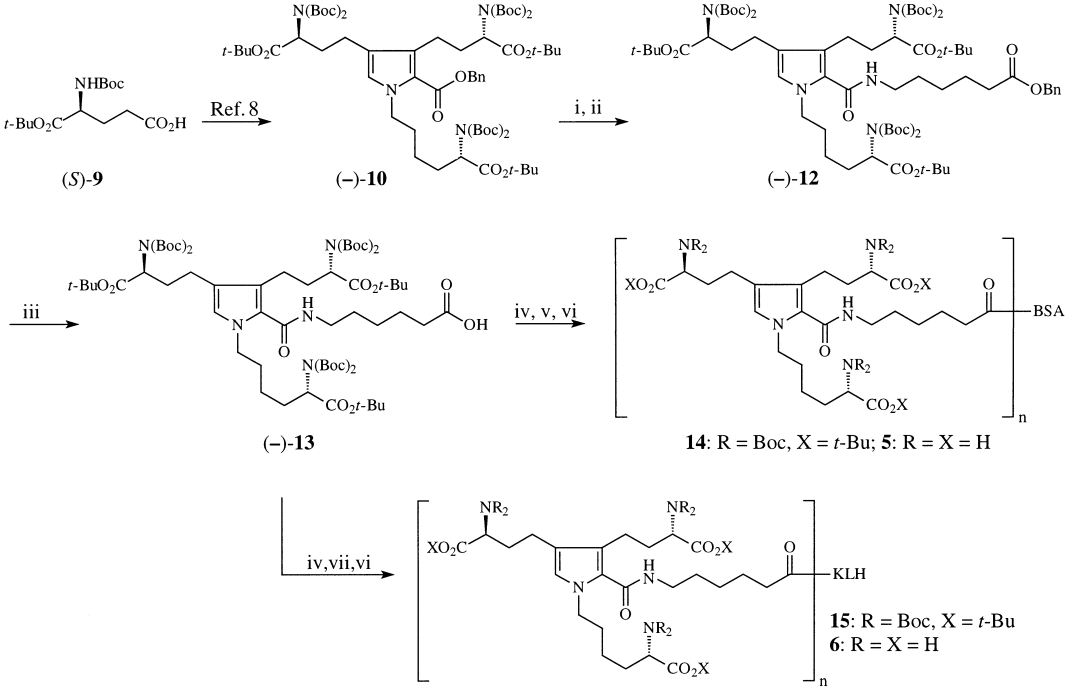
1: X = OH, Pyridinoline
2: X = H, Deoxypyridinoline
3: X = OH, Pyrrololine
4: X = H, Deoxypyrrololine

Figure 1.

alternative hapten-carrier protein conjugate such as KLH-immunogen, is required to select the *anti*-analyte antibodies. Therefore, the key hapten, (−)-**13**, a modified Dpl (**4**) with a linking arm 2-position of pyrrole ring, was envisioned from (−)-ester (**10**).

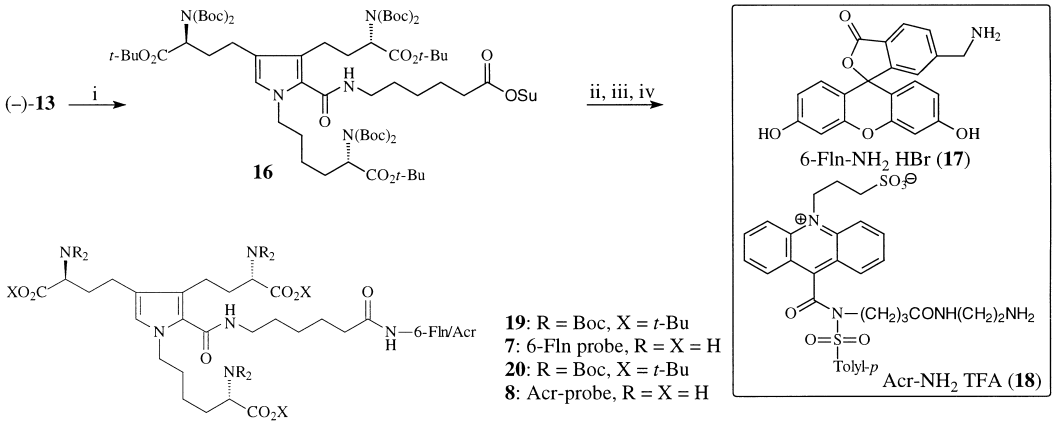
Accordingly, the (−)-benzyl ester (**10**), which was prepared from glutamic acid derivative (*S*)-**9**,[9–11] upon hydrogenolysis, and activation using (Scheme 1) HOSu gave the corresponding succinimidyl ester, which was reacted with benzyl-6-aminohexanoate (**11**) in the presence Et_3N to afford (−)-**12** in 74% yield. The benzyl group in (−)-**12** was removed by hydrogenation over Pd/C in ethanol to give the key hapten (−)-**13** in 97% yield. The acid (−)-**13** was activated with HOSu and EDAC in CH_2Cl_2 and conjugated to BSA in THF and buffer (pH: 8.0) at room temperature. After 24 h, the crude reaction mixture was purified by dialysis to remove the hydrolyzed hapten (−)-**13** and other low molecular weight impurities. Lyophilization of the product gave an immunogen precursor (**14**) in which the amino groups were unmasked using trifluoroacetic acid and water. The immunogen **5** was purified by dialysis and obtained as a white powder after lyophilization. Analysis of immunogen **5** by gel electrophoresis[20] showed a single narrow band indicating its homogeneity and the absence of free and polymeric BSA. The resolution of gel electrophoresis is, however, inadequate to determine the hapten density. Therefore, the immunogen **5** was analyzed by MALDI[21] and its molecular weight was found to be 81,503, which represents an average of 27 haptens incorporated per BSA. The immunogen **6** was also prepared from (−)-**13** and KLH by following the procedure developed for **5**.

Fluorescence polarization immunoassay (FPIA)[22] and chemiluminescence immunoassay (CLIA)[23,24] are two commonly used immunoassay clinical systems for measurement of analytes. These technologies require fluorescent and chemiluminescent signal generating probes, respectively, which are commonly referred to as tracers. In order to allow the tracer to mimic overall topology of the hapten, which was used to prepare the immunogen, the fluorescent and chemilumimescent labels were directly attached to (−)-hapten **13**. Thus, (−)-**13** was activated (Scheme 2) to give the corresponding succinimidyl ester (−)-**16**, which was treated with 6-aminomethylfluorescein hydrobromide (**17**)[25] in DMF and in the presence of Et_3N to afford **19** in 69% yield. Hydrolysis of the Boc and *t*-butyl esters groups in **19** using trifluoroacetic acid followed by reversed phase (RP) HPLC purification afforded the 6-fluorescent probe (6-Fln-probe, **7**) in 84% yield and >99% purity.[26] Similarly, the active ester (−)-**16** was treated with acridinium derivative (**18**)[27] to afford **20** in 72% yield after purification by HPLC. Finally, hydrolysis of **20** using



14: R = Boc, X = *t*-Bu; **5**: R = X = H
15: R = Boc, X = *t*-Bu
6: R = X = H

Scheme 1. Reagents and conditions: (i) 10% Pd/C, EtOH, H_2, rt, 1 h 95%; (ii) HOSu, EDAC, CH_2Cl_2, rt, 4 h, then $BnO_2C(CH_2)_6NH_2$ (**11**), Et_3N, CH_2Cl_2, rt, 21 h, 74%; (iii) 10% Pd/C, EtOH, H_2, rt, 1 h, 97%; (iv) HOSu, EDAC, CH_2Cl_2, rt, 17 h; then (v) BSA, THF, buffer (pH: 8.0), rt, 24 h; (vi) TFA, H_2O, (ratio: 9.5:0.5), rt, 2 h; (vii) KLH, THF, buffer (pH: 8.0), rt, 24 h.



Scheme 2. Reagents and conditions: (i) HOSu, EDAC, CH_2Cl_2, rt, 16 h 72%; (ii) **17**, DMF, Et_3N, rt, 16 h (60%); (iii) **18**, DMF, Et_3N, rt, 16 h, (80%); (iv) TFA–H_2O (9.5:0.5 ratio), rt, 1.5 h (50, 58%).

trifluoroacetic acid followed by HPLC purification afforded the chemiluminescent probe (Acr-probe, **8**) in 77% yield and >99% purity as a yellow powder.[28]

In summary, two site specifically derivatized immunogens (**5**,**6**) and two probes (fluorescent **7**, chemiluminescent **8**) were prepared from (−)-hapten **13** for generation of *anti*-Dpl antibodies and development assays for osteoporosis.

References and Notes

1. *Principles of Bone Biology*; Bilezikan, J. P., Raicz, L. G., Rogan, G., Eds.; Academic Press: New York, 1996.
2. Knott, L.; Baily, A. J. *Bone* **1998**, *22*, 181–187.
3. James, I. T.; Walne, A. J.; Perrett, D. *Ann. Clin. Biochem.* **1996**, *33*, 397–420.
4. Rosano, T. G.; Peaston, R. T.; Bone, H. G.; Woitge, H. W.; Francis, R. M.; Seibel, M. *J. Clin. Chem.* **1998**, *44*, 2126–2132.
5. Scott, J. E.; Hughes, E. W.; Shuttleworth, A. A. *Biosci. Rep.* **1981**, *1*, 611–618.
6. Kuypers, R.; Tyler, M.; Kurth, L. B.; Jenkins, I. D.; Horgan, D. J. *J. Biochem.* **1992**, *283*, 129–136.
7. Hanson, A. A.; Eyre, D. R. *J. Biol. Chem.* **1996**, *271*, 26508–26516.
8. Adamczyk, M.; Johnson, D. D.; Reddy, R. E. *Angew. Chem., Int. Ed.* **1999**, *38*, 3537–3539.
9. Adamczyk, M.; Johnson, D. D.; Reddy, R. E. *Tetrahedron* **1999**, *55*, 63–88.
10. Adamczyk, M.; Johnson, D. D.; Reddy, R. E. *Tetrahedron Asymmetry* **1999**, *10*, 775–781.
11. Adamczyk, M.; Akireddy, S. R.; Reddy, R. E. *Tetrahedron Asymmetry* **1999**, *10*, 3107–3110.
12. Hage, D. S. *Anal. Chem.* **1999**, *71*, 294R–304R.
13. Sokoll, L. J.; Chan, D. W. *Anal. Chem.* **1999**, *71*, 356R–362R.
14. Wild, D. *The Immunoassay Handbook*; Stockton Press: New York, 1994.
15. *Antibodies: A Laboratory Manual*; Harlow, E., Lane, D. Eds.; Cold Spring Harbor: New York, 1988.
16. Adamczyk, M.; Fishpaugh, J.; Harrington, C.; Hartter, D.; Johnson, D.; Vanderbilt, A. *J. Immunol. Methods* **1993**, *162*, 47–58.
17. Adamczyk, M.; Fishpaugh, J.; Harrington, C.; Johnson, D.; Vanderbilt, A. *J. Immunol. Methods* **1993**, *163*, 187–197.
18. Adamczyk, M.; Gebler, J. C.; Gunasekera, A. H.; Mattingly, P. G.; Pan, Y. *Bioconjugate Chem.* **1997**, *8*, 133–145.
19. Adamczyk, M.; Johnson, D. D.; Mattingly, P. G.; Moore, J. A.; Pan, P. *Bioconjugate Chem.* **1998**, *9*, 23–32.
20. Kamps-Holtzapple, C.; Carlin, R. J.; Sheffield, C.; Kubena, L.; Stanker, L.; DeLoach, J. R. *J. Immunol. Methods* **1993**, *164*, 245–253.
21. Adamczyk, M.; Buko, A.; Chen, Y.-Y.; Fishpaugh, J.; Gebler, J. C.; Johnson, D. D. *Bioconjugate Chem.* **1994**, *5*, 631–635.
22. Dandliker, W. B.; Hsu, M.-L.; Vanderlaan, W. P. In *Immunoassays: Clinical Laboratory Techniques for the 1980's*, Nakamura, R. M., Dito, W. R., Tucker, E. S. Eds.; Alan R. Liss: New York, 1980, pp 89–126.
23. Dudley, R. F. *Lab. Medicine* **1990**, *21*, 216–222.
24. Jandreski, M. A. *Lab. Medicine* **1998**, *29*, 555–560.
25. 6-Fln-NH_2 HCl is available from Molecular Probes, Inc., Eugene, OR.
26. Purified by preparative reversed phase (RP) HPLC (Waters, RCM, μBondpak C18, 10 μm, 40 × 100 mm) using MeCN:0.1% aq trifluoroacetic acid 18:82, 45.0 mL/min at 215 nm. Analytical RP HPLC (Waters, RCM, μBondpak C18, 10 μm, 8 × 100 mm): MeCN:0.1% aq trifluoroacetic acid 20:80, 2.0 mL/min at 215 nm; R_t: 9.40 min, 96.3%; ^{1}H NMR (D_2O): δ 8.12–8.06 (br s, 1H), 7.74–7.66 (br s, 1H), 7.36 (s, 1H), 6.95–6.87 (m, 2H), 6.73–6.64 (m, 3H), 6.45 (s, 2H), 4.54 (s, 2H), 3.96–3.72 (m, 5H), 3.14–3.04 (m, 2H), 2.68–1.96 (m, 10H), 1.80–1.02 (m, 12H); ESI–MS (*m*/*z*): 899 $(M)^+$.
27. Mattingly, P. G. *J. Biolumin. Chemilumin.* **1991**, *6*, 107–114.
28. Purified by preparative reversed phase (RP) HPLC (Waters, RCM, Bondpak C18, 10 μm, 40 × 100 mm) using MeOH:0.1% aq trifluoroacetic acid: 43:57, 25.0 mL/min at 215 nm. Analytical RP HPLC (Waters, RCM, μBondpak C18, 10 μm, 8 × 100 mm): MeOH:0.1% aq trifluoroacetic acid: 45:55, 1.0 mL/min at 215 nm; R_t: 8.68 min, 99.7%; ^{1}H NMR ($D_2O + CD_3OD$): δ 8.74–7.58 (m, 8H), 7.00–6.61 (m, 5H), 5.64–5.54 (m, 2H), 4.36–4.28 (m, 2H), 3.98–3.76 (m, 5H), 3.50–3.18 (m, 8H), 2.89–1.86 (m, 16H), 1.84–1.02 (m, 16H); ESI–MS (*m*/*z*): 1165 $(M)^+$.