Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 22, pp. 2831-2836, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)10084-1

FIRST TOTAL SYNTHESIS OF THE BONE RESORPTION MARKERS DEOXYPYRIDINOLINE AND HYDROXYPYRIDINOLINE

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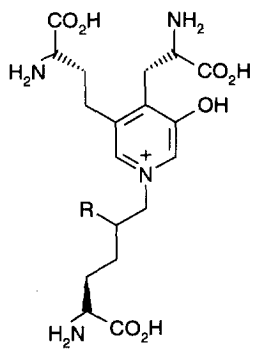
Abstract: The first total synthesis of the collagen crosslinks deoxypyridinoline **1** and hydroxypyridinoline **2** was achieved. The key intermediate, pyridine **3** served as starting material for the preparation of compounds **1** and **2** and derivatives of **1**. These compounds create optimal tools to establish a diagnostic kit for bone resorption. © 1997 Elsevier Science Ltd.

Introduction

The collagen crosslinks deoxypyridinoline (DP) **1** and hydroxypyridinoline (HP) **2** are two markers for bone resorption, especially DP which only occurs in bone and dentine[1]. Suitable markers for bone resorption are important tools in the early diagnosis and drug therapy monitoring in patients with metabolic bone disease. So far these two collagen crosslinks were isolated from urine, using several cleaning steps and in low yields[2]. To overcome this pitfall we initiated the chemical synthesis of DP and HP. Furthermore we were interested in the preparation of DP derivatives with well defined coupling sites for bovine serum albumin (BSA) and other proteins which can be used to generate antibodies against DP.



R: H, DP (**1**)
R: OH, HP (**2**)

As a result of retrosynthetic analysis (see Scheme 1) it became clear that the pyridine-derivative **3** will be the key intermediate of the synthesis. Starting from compound **3** both DP (**1**) and HP (**2**) and derivatives of **1** could be prepared by introduction of the corresponding side chains (e.g. **4** or **5**). In nature, Pyridine **3** is formed from two lysine containing compounds[3], therefore we planned to prepare this compound starting from epoxy-amino acid **6** in a biomimetic way.

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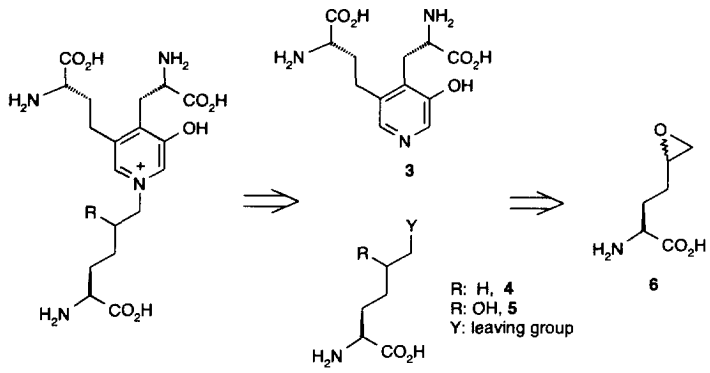
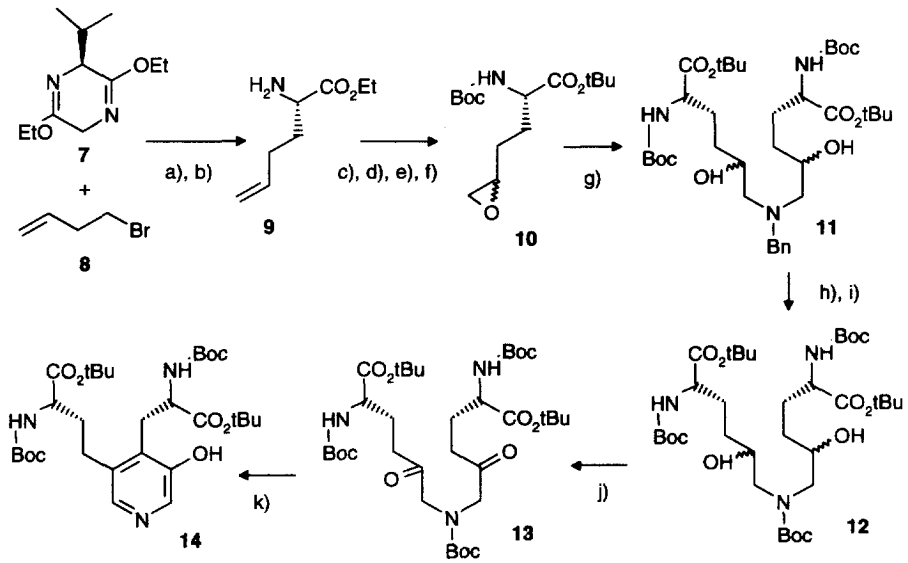
Scheme 1



As protecting groups in all the following synthesis tert. butoxycarbonyl (Boc) was used for the amino group and tert. butyl (tBu) for the carboxylic acid group. This choice allowed us to deprotect all derivatives by trifluoroacetic acid (TFA)/water 95:5 at the end of the synthesis. Using different protecting groups and therefore different cleaving conditions made the purification of the final products very difficult and gave very poor yields. In fully protected form all compounds could be purified with classical column chromatography on silica gel.

Scheme 2



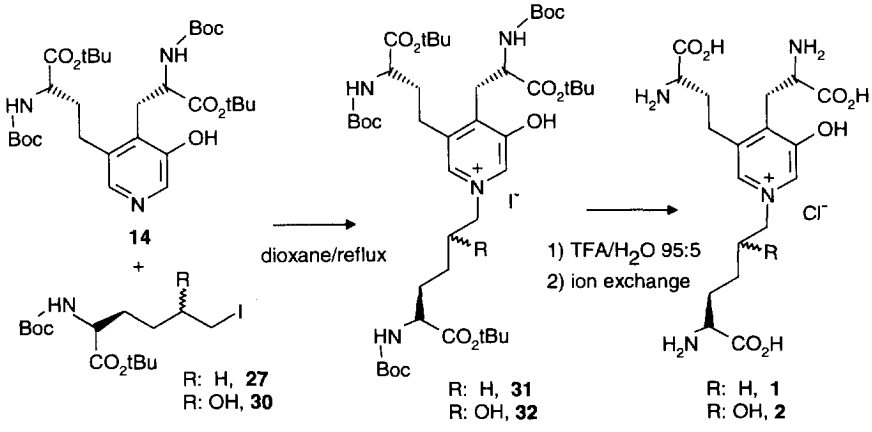
a) *n*-BuLi; b) 0.25 N HCl; c) $(Boc)_2O$; d) 1N NaOH; e) EDCI, DMAP, *t*-BuOH; f) mCPBA g) Bz-NH_2, reflux; h) H_2/Pd/C; i) $(Boc)_2O$; j) Swern Oxidation; k) DBU.

Chemical Synthesis

For the preparation of the fully protected L-α-amino acid **6**, Schoellkopf's approach was used[4]. Therefore, starting with bislactim ether **7** and 4-bromo-1-butene **8** following the protocol of Schoellkopf, the L-α amino acid **9** was prepared. Boc protection of the amino group and transesterification of the carboxylic acid group followed by epoxidation with meta chloroperbenzoic acid (mCPBA) gives the desired epoxy-amino acid derivative **10**. Treatment of epoxide **10** with 0.5 eq. of benzylamine at 65° C over night without any solvent results in compound **11**. Hydrogenation of **11** in the presence of 10% palladium on carbon followed by Boc protection of the resulting amine gave compound **12** in nearly quantitative yield. Swern oxidation protocol was the best for the conversion of the dialcohol **12** to diketone **13**. The cyclisation of diketone **13** to pyridine **14** was performed in a open vessel in the presence of 1,8-diazabicyclo(5,4,0)undec-7-en(1.5-5) (DBU) at room temperature for 24 h. So far this reaction works only with DBU in reasonable yields (50 - 60%)[5] (Scheme 2).

With the fully protected key intermediate **14** in hand, we were able to prepare fully protected **31** (Yield 70%) and **32** (Yield 65%) just by heating pyridine **14** and the appropriate iodide **27** or **30** in dioxane for 1 hour (Scheme 3).

Scheme 3

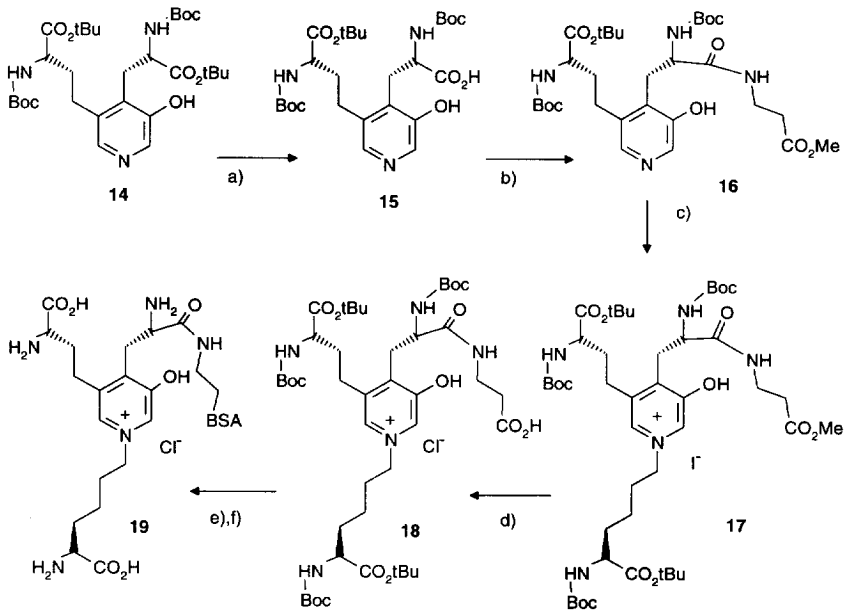


Deprotection of derivatives **31** and **32** with trifluoroacetic acid/water 95:5 mixture gave after purification by preparative HPLC and ion exchange chromatography the chloride salt of either DP **1** or HP **2** (Scheme 4). Both compounds were identical with respect to mass spectra, ^{1}H NMR spectroscopy and HPLC compared to natural products isolated from urine[6,7].

Starting from pyridine **14** we were able to prepare compound **15** be treatment with 10 equivalent 2N sodium hydroxide (NaOH). Interestingly only the tert. butylester of the "short chain" amino acid was cleaved by treatment with 2N NaOH. We explain this observation by the neighbouring group participation of the hydroxyl group on the pyridine ring. To compound **15** β-alanine methylester was coupled using standard coupling procedure to get compound **16**. Introduction of the side chain as described above gave derivative **17**.

Saponification of **17** followed by coupling of BSA to the free acid gives compound **18**. Deprotection of this derivative using conditions as described above gives the DP-BSA derivative **19**.

Scheme 4



a) 2N NaOH; b) β-H-Ala-OMe/DCC/HOBT; c) iodide **27**/dioxane/reflux; d) 1N NaOH; e) *N*-hydroxysuccinimide/BSA/puffer pH=8; f) TFA/H_2O 95:5.

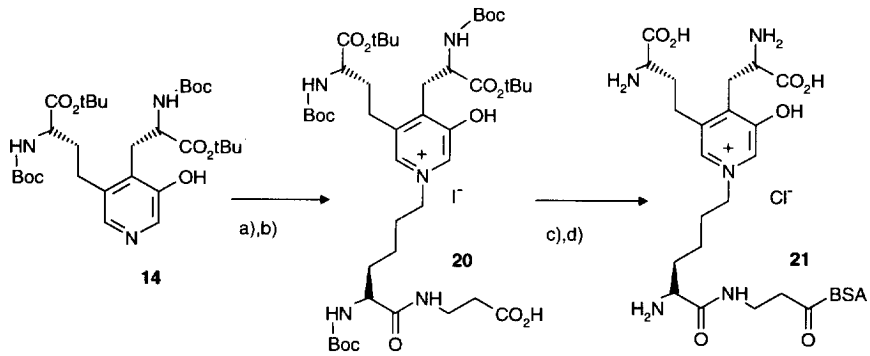
In Scheme 5 we describe the synthesis of a the DP-BSA complex **21** in which BSA is coupled to the "longest chain" amino acid, using the same reaction conditions as in the previous reaction protocols.

On the same positions as BSA (compounds **19** and **21**) we coupled also Hemocyanin from Keyhole Limpets (KLH), derivatives of biotin and derivatives of tyrosine using the described synthetic strategies. To proof binding of DP onto BSA, part of compound **19** and **21** were cleaved with classical peptide degradation methods. The remaining amino acids were analysed, and DP was found, by comparison with synthetic and natural DP by HPLC methods. Afterwards with these two preparations immunisation was started.

Discussion

The described synthesis allow us not only to prepare DP and HP in high quality but also to manipulate each carboxylic acid function within DP separately and very easily. This makes it possible to prepare new DP-BSA complexes, in which β-alanine is replaced by an other spacer molecule. Therefore we are able to prepare alternative DP-BSA derivatives, if the complexes **19** and/or **21** produce antibodies against DP which do not fulfil our requirements.

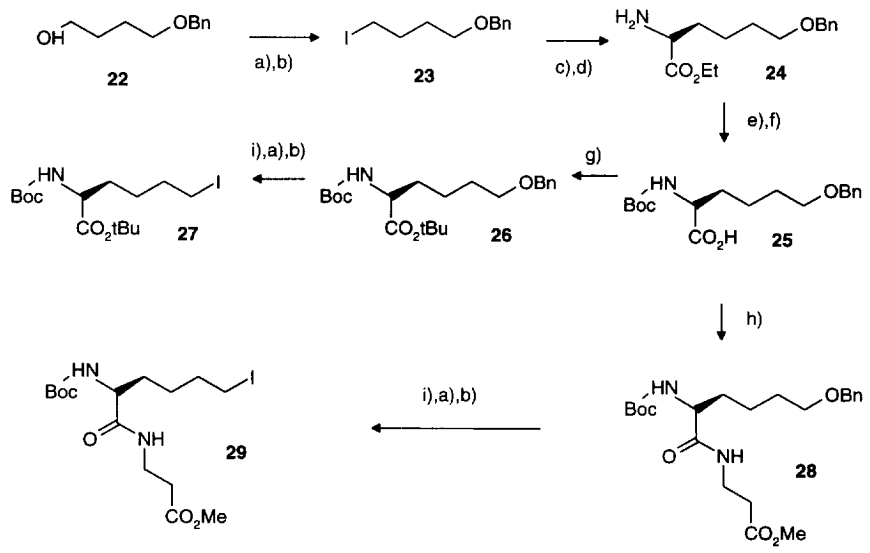
Scheme 5



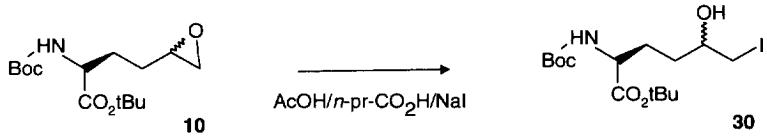
a) iodide **29**/dioxane/reflux; b) 1N NaOH; c) *N*-hydroxysuccinimide/BSA/puffer pH 8; d) TFA/H_2O 95:5

In scheme 6 the synthesis of the iodides which were used to introduce the different side chains onto pyridine **14** is outlined. All synthesis steps are straight forward and proceed in good to excellent yields.

Scheme 6



a) TsCl; b) NaI; c) bislactim ether **7**/*n*-BuLi; d) 0.25N HCl; e) $(Boc)_2O$; f) 1N NaOH; g) EDCI/DMAP/*t*-BuOH; h) β-H-Ala-OMe/DCC/HOBT; i) H_2/Pd/C.



Conclusions

This unique situation of having chemically-defined DP and HP standards will allow us to measure bone turnover under well standardised conditions using classical HPLC methods. In addition we have created the optimal tools, in having the possibility to prepare well-defined antigen-DP-complexes from our synthetic intermediates, to establish an easily accessible diagnostic kit for bone resorption[8]. With these synthetic intermediates we may have an advantage over others (e.g. crossreactivty over HP), who have to prepare antibodies against DP with preparations which were isolated from natural sources. Immunisation with our synthetic PD-antigen complexes and development of a kit are under investigation.

Acknowledgements: The authors wish to thank Ms. T. Zardin for the measurement and interpretation of ^{1}H-NMR spectra and Ch. Quiquerez for the measurement and interpretation of mass spectra.

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5. For an alternative synthesis of highly substituted 3-hydroxy-pyridin derivatives see also: Doktorova, N.D.; Ionova, L.V.; Karpeisky, M.Ya.; Padyukova, N.Sh.; Turchin, K.F.; Florentiev, V.L. *Tetrahedron* **1969**, *25*, 3527.
6. For isolation and HPLC measurement a similar procedure as described by Black, D.; Duncan, A.; Robins, S.P. *Anal. Biochem.* **1988**, *169*, 197 was used.
7. Preparation of **1**: 206 mg (0.2 mMol) of **31** were dissolved in 20 ml of 95% trifluoro acetic acid/water and stirred for 90 min. at room temperature. The solvent was evaporated and the residue was dissolved in 2 ml methanol and 0.5 ml 2N hydrochloric acid (in diethylether) and evaporated again. The residue was separated by HPLC on a Macherey/Nagel SA5 column (200x4.6 mm SCX, 5µm). PufferA, 0.02 M LiCl, pH 3.1; PufferB, 0.2 M LiCl, pH 9.5; Gradient 0-100% B, 50 min.; Flow rate 1 mL/min. Fractions containing **1** were collected and evaporated and desalted twice over a Bio-Gel P-2 column, Cl^- free fractions (silver nitrate test) were pooled and lyophilised to give **1** as a white solid in 10 % yield. ^{1}H NMR (D_2O, 360 MHz) δ 1.35-1.55 (m, 2H), 1.85-1.95 (m, 2H), 2.00-2.10 (m, 2H), 2.10-2.25 (m, 2H), 2.80-3.05 (m, 2H), 3.35-3.45 (m, 2H), 3.80 (t, 1H), 3.90 (t, 1H), 4.10-4.15 (m, 1H), 4.50 (t, 2H), 8.20 (s, 1H), 8.30 (s, 1H). FAB MS *m/e* 413 ($[M]^+$).
8. Uebelhart, D., Gineyts, E., Chapuy, M.-C., Delmas, P.D., *Bone and Mineral*, 1990, 8, 88.

(*Received in Belgium* 3 *September* 1997; *accepted* 2 *October* 1997)