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SYNTHESIS OF REDUCED COLLAGEN CROSSLINKS

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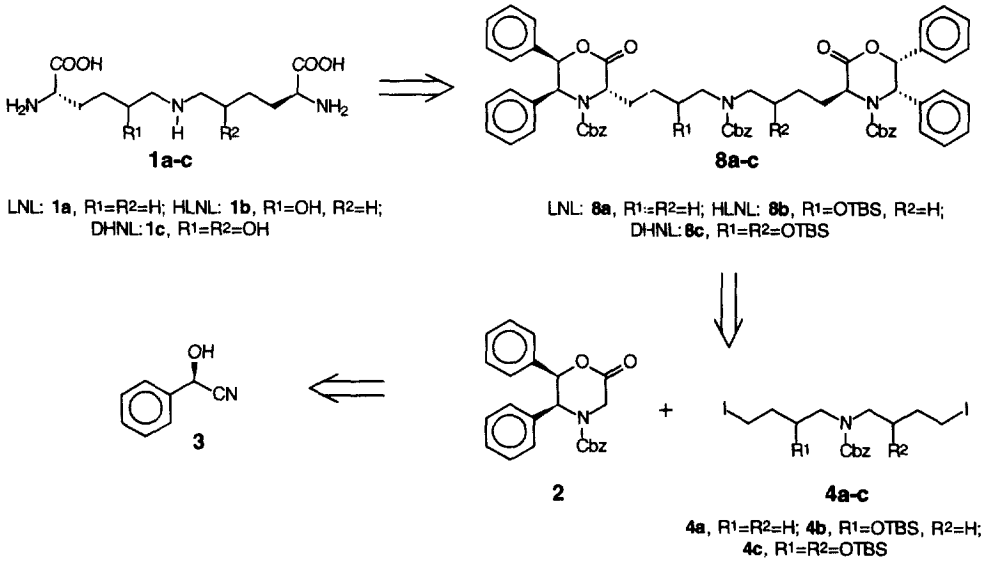
Abstract: A new synthetic route to reduced collagen crosslinks (LNL and HLNL) is described in this report. It enables an enantioselective synthesis of LNL. HLNL was obtained as a mixture of two diastereoisomers. This method also provides the possibility to introduce radio-labels during the synthesis. © 1999 Elsevier Science Ltd. All rights reserved.

Collagen is the major structural protein in the human body. It is a key molecule for the mechanical strength of tissues like bone, cartilage, skin, and tendon. Triple helical collagen molecules are organized in collagen fibers. In particular the intermolecular crosslinks provide the fiber with its high tensile strength. In the human body this crosslinking is initiated by enzymatic oxidation of lysine or hydroxylysine residues to aldehydes, followed by condensation with a second lysine or hydroxylysine residue. Stabilisation by reduction is required for the bifunctional crosslinks to be isolated from human tissue. The reduced crosslinks have been identified as lysinonorleucine (LNL, **1a**), 5-hydroxy-lysinonorleucine (HLNL, **1b**) and 5,5'-dihydroxylysinonorleucine (DHLNL, **1c**). As these amino acids are naturally occurring, the α-amino centers possess the *S*-configuration. At present, the configuration at the hydroxylated centers is unknown[1].



***Scheme 1:** Retro-synthetic analysis for the synthesis of collagen crosslinks.*

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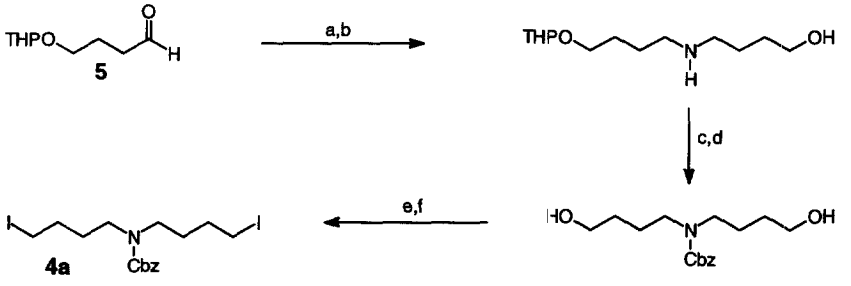
An earlier described synthesis for these bifunctional crosslinks[2] lacks the opportunity for an enantioselective approach. The present study was initiated to enable the stereoselective synthesis of these crosslinks. The synthesized materials can serve as references in the analysis of reduced collagen crosslinks by reversed-phase HPLC.

Our convergent synthetic approach is illustrated by the retro-synthetic analysis presented in Scheme 1 and depends heavily on the use of the Williams Glycine Template (**2**) for asymmetric α-amino acid synthesis[3]. Compound **2** is commercially available[4], and has recently become available on multigram scale in enantiomerically pure form by an efficient stereoselective synthesis from *(R)*-mandelonitrile (**3**)[5].

Results and Discussion

First, the secondary amine backbones **4** of the crosslinks had to be constructed and provided with iodine substituents at both ends of the chain. In case of LNL this was accomplished by the straightforward synthetic route depicted in Scheme 2. Aldehyde **5**[6] was converted into diiodide **4a** via a sequence of well known reactions in 71% overall yield.



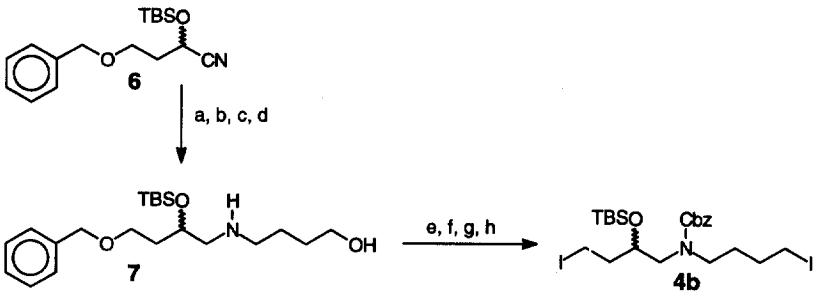
a) 4-amino-1-butanol b) $NaBH_4$ c) p-TSA / MeOH d) Cbz-Cl e) Ts-Cl f) NaI / acetone

***Scheme 2**: Synthesis of the secondary amine backbone for LNL.*

For the HLNL-backbone the protected cyanohydrin **6** served as the starting material[7] (Scheme 3). Via a three step, one pot, *DIBAL reduction-transimination-hydride reduction* sequence developed earlier[8], the racemic secondary amine **7** was obtained in almost quantitative yield. Transformation of amine **7** into diiodide **4b** proceeded relatively simply in 40% overall yield. In both routes to **4a** and **4b** an imine-intermediate is involved. At this imine stage a label, as in the work up of the natural material, can be introduced. For instance deuterium or tritium via a $NaBD_4$ or $NaBT_4$ reduction, or a cyanide via *a DIBAL reduction-transimination-cyanide addition* reaction reported by Zandbergen et al.[9]

Alkylations of the Williams Glycine Template (**2**) were carried out using experimental details described by Baldwin et al.[10] Thus, generation of the enolate of **2** (2 eq) in THF, using sodium bis(trimethylsilyl)amide (2 eq) as the base at -80 ^{0}C in the presence of 15-crown-5 (6 eq), followed by the addition of diiodide **4a** (1 eq), stirring at -80 ^{0}C for 30 minutes and slowly warming to room temperature yielded the dialkylated precursor **8a** ($[\alpha]^{20}_D$ -22 (c = 0.5, CH_2Cl_2)) in 50% yield after column chromatography. A considerable amount (26%) of mono-alkylated iodide was also isolated.

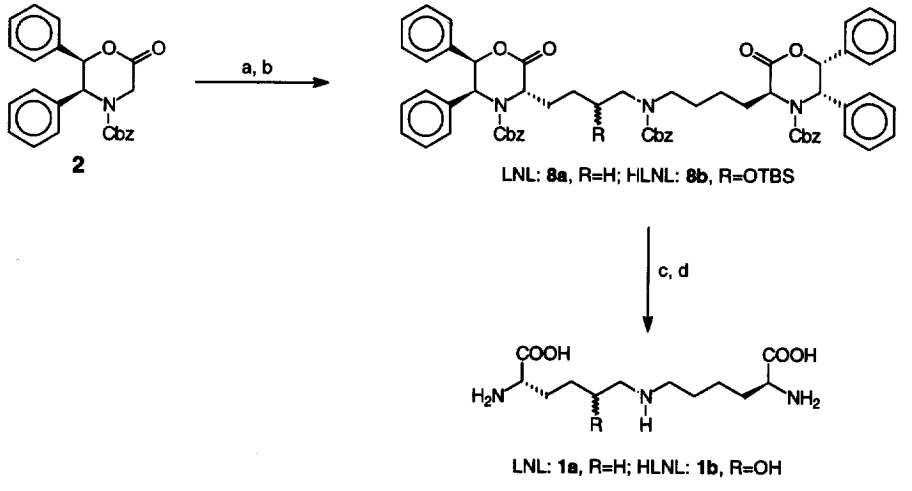
Characterization was achieved using 300 MHz 2D NMR. Only a single, symmetrical, diastereoisomer was observed, which implicates a diastereomeric purity, and therefore in this case (**8a**) also an enantiomeric purity, of > 95%.



a) DIBAL b) MeOH c) 4-amino-1-butanol d) $NaBH_4$ e) Li / NH_3 f) Cbz-Cl g) Ts-Cl h) NaI / acetone

Scheme 3: Synthesis of secondary amine backbone for HLNL.

In a similar fashion, precursor **8b** was obtained in 32% yield. When compound **8b** was isolated it was anticipated that the two diastereoisomers would be separable and thus lead to both possible enantiomeric crosslinks **1b**. However on TLC no separation was observed and also by NMR the diastereoisomers were not distinguishable. It was therefore concluded that the hydroxylated center was too far away from the other stereogenic centers to be of any influence in chromatographic and NMR-spectroscopic behavior.



a) $NaN(TMS)_2$ b) diiodide **4a** or **4b** c) TBAF (case **8b**) d) $Pd(OH)_2$ / H_2

*Scheme 4: Double Alkylation of diiodides **4a** and **4b** with the Williams Glycine Template to obtain the crosslink precursors **8a** and **8b** which, after deprotection, afforded the reduced collagen crosslinks **1a** and **1b**.*

Reductive removal of all seven benzylic protecting groups was achieved by catalytic hydrogenation using $Pd(OH)_2$ (20% on activated carbon) as the catalyst. Trituration with dry ether to remove 1,2-diphenylethane (side product from the deprotection), filtration through a SEP PAK C18 cartridge and lyophilization afforded LNL (**1a**) as a white solid in 95% yield. HLNL was obtained after removal of the TBS-group with TBAF (70-80% yield) and reductive cleavage of the benzylic positions under identical conditions as described for LNL. The reduction afforded, after purification, HLNL as a mixture of two diastereoisomers in 72% yield.

Both LNL and HLNL were characterized by 300 MHz 2D NMR[11] and exact mass spectroscopy[12]. For further analysis LNL was converted into its diethyl ester[13] and mass spectrometry was in agreement with literature[13]. Derivatization of HLNL and LNL with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by analytical reversed-phase HPLC[14] resulted in single peaks[15] indicating a purity of at least 95% for both compounds.

In conclusion, a useful method to obtain enantiomerically pure LNL has been developed. HLNL was obtained as a mixture of two diastereoisomers. Future research will focus on the complete enantioselective synthesis of HLNL and DHLNL and elucidation of the configuration of the hydroxylated centers in these collagen crosslinks. An enantioselective synthesis of cyanohydrin **6**, a key intermediate in the route described above, is expected to open the way to all possible stereoisomers of HLNL and DHLNL.

References and Notes

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6. Aldehyde **5** was prepared in 70% yield from 1,4-butanediol via protection as the mono-THP ether and subsequent PCC oxidation to the aldehyde.
7. Cyanohydrin **6** was prepared from 1,3-propanediol via protection as the mono-benzyl ether, PCC oxidation to the aldehyde followed by HCN addition and protection of the cyanohydrin with TBS-Cl. (54% overall yield).
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11. a) LNL: (0.1M DCl, δ in ppm) ^{1}H NMR; 1.51 (m, 4H), 1.77 (m, 4H), 1.94 (m, 4H), 3.10 (t, 4H, *J*=7.7 Hz), 3.80 (dd, 2H, *J*=*J*=6.1 Hz). ^{13}C NMR; 22.6, 26.1, 30.6, 48.2, 55.8, 173.2.
    b) HLNL: (0.1M DCl, δ in ppm) ^{1}H NMR; 1.52 (m, 4H), 1.77 (m, 2H), 1.96 (m, 4H), 3.10 (m, 4H), 3.98 (m, 3H). ^{13}C NMR; 22.1, 25.5, 26.7, 30.0, 30.1, 30.2, 47.8, 52.7, 53.8, 66.8, 173.2, 173.3.
12. Exact Mass experiments(ESI): a) LNL: M+H 276.18773; theoretical 276.18873, b) HLNL: M+H 292.17875; theoretical 292.17941, c) LNL diethyl ester: 332.25344; theoretical 332.25493.
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15. Retention Times(min): HLNL 26.23, LNL 27.45, hydroxylysine 24.07, lysine 25.40.