Synthesis of Anabasine-6'-carboxylic Acid from Aldosine, the Aldol Crosslinking Amino Acid of Elastin

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A novel pyridine compound, 6-(3-pyridyl)pipecolinic acid **2**, was synthesized by treatment, with Fe^{3+}/activated charcoal, of aldosine **1** which was the acid hydrolysis product of the aldol crosslink moiety **3** of elastin. Structural identification was performed by spectroscopic analysis. Compound **2** was named anabasine-6'-carboxylic acid, because it is the 6'-carboxylic acid derivative of anabasine, which is an alkaloid of (among others) the tobacco plant. Anabasine-6'-carboxylic acid might be an oxidative decarboxylation product of aldosine.

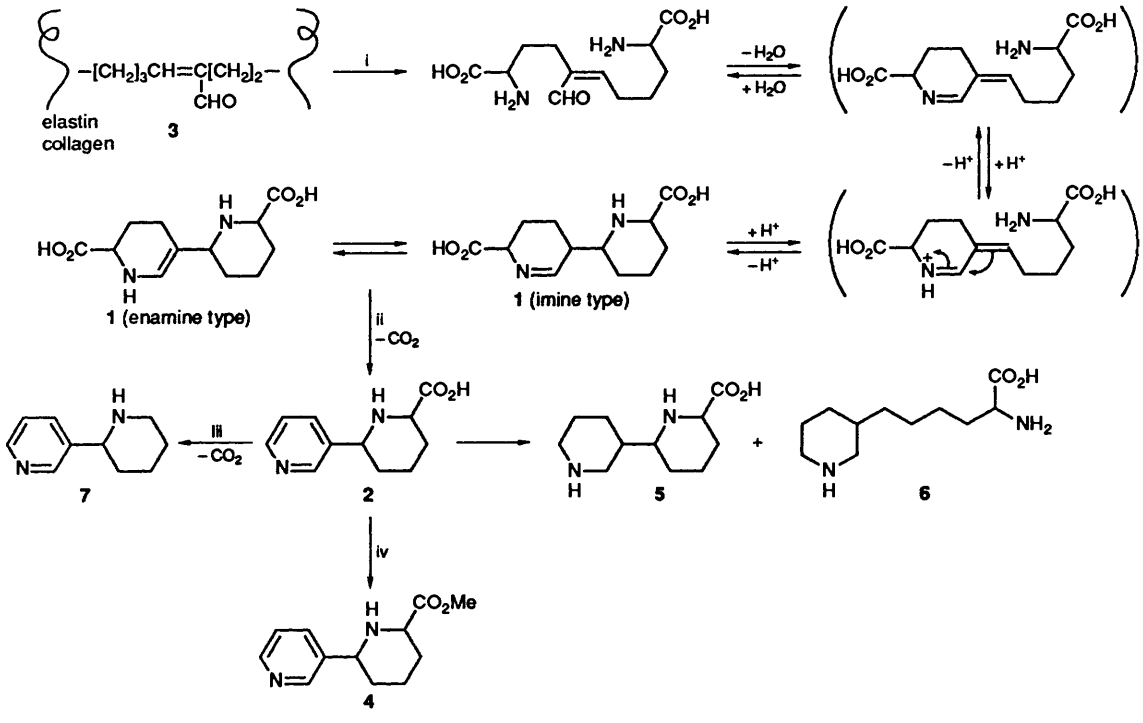
Elastin is a connective tissue protein found in virtually all tissues and organs of mature animals and is a major constituent of blood vessels, lungs and ligaments. Elastin is primarily synthesized during physiological development by such cells as vascular smooth muscles and fibroblasts *via* a soluble precursor, tropoelastin.[1] Tropoelastin is exposed to the extracellular space where it continues to undergo chemical modifications that ultimately result in formation of specific kinds of covalent attachments called crosslinks. Crosslinking principally involves residues of lysine that undergo oxidative transformation to aldehyde (α-aminoadipic acid δ-semialdehyde; allysine); in turn, these engage other residues in the formation of aldol and other types of condensation products as shown in Scheme 1.

A variety of crosslinking amino acids have been isolated from elastin hydrolysis products and have been identified.[2] Among these, aldol crosslink moiety **3** is the predominant crosslink that is present in elastin. In addition, a family of many types of different crosslinks results from moiety **3**. Desmosine[3] and isodesmosine[3] are tetrafunctional pyridinium crosslinking amino acids, and allodesmosine[4] and pentasine[5] are pentafunctional pyridinium crosslinking amino acids.

Recently we reported that a new amino acid, which we named aldosine **1**, was isolated from acid hydrolysis products of bovine elastin and collagen and was found to be formed from the crosslinking moiety **3** of such proteins.[6] As aldosine **1** has a highly reactive and unique structure, we have recently initiated a programme to investigate the synthesis of bioactive compounds from aldosine **1**. In this paper we report a synthesis of anabasine-6'-carboxylic acid **2** from aldosine **1** isolated from acid hydrolysates of bovine ligamentum nuchae elastin by oxidative decarboxylation using Fe^{3+}/activated charcoal. Formation of anabasine **7** by thermal decarboxylation of compound **2** is also described.

Results and Discussion

Separation of Aldosine **1**.—Elastin from bovine ligamentum nuchae was hydrolysed by 6 mol dm^{-3} HCl in the conventional



Scheme 1 *Reagents and conditions:* i, acid hydrolysis; ii, O_2, Fe^{3+}/activated charcoal; iii, heat; iv, MeOH, HCl

Table 1 ^{13}C and ^{1}H NMR data for compound **2** in D_2O

Atom	δ_C	δ_H[mult, J (Hz), integration]
1′ (N)		
2′	143.67	8.96 (1 H, s)
3′	138.52	
4′	148.98	8.76 (1 H, d, 8.4)
5′	130.89	8.15 (1 H, dd, 8.1, 5.9)
6′	145.02	8.85 (1 H, d, 5.5)
1 (N)		
2	61.43	4.23 (1 H, dd, 12.1, 3.3)
3	27.64	1.85a (1 H, m)
		2.40e (1 H, m)
4	24.73	1.85a (1 H, m)
		2.10e (1 H, m)
5	30.57	2.15 (2 H, m)
6	59.94	4.70 (1 H, dd, 12.5, 2.9)
7	173.47	

a = axial, e = equatorial.

manner at 110 °C for 48 h. The acid hydrolysis products of elastin were charged on a column of activated charcoal which had been reduced by treatment with borohydride. Major lysine-derived crosslinking amino acids containing aldosine **1** which was derived from crosslink moiety **3** by acid hydrolysis were fractionated from water, followed by elution with 50% aq. methanol. The 50% aq. methanol fraction was evaporated to give a syrup and this was then charged on a silica gel column with ethyl acetate–acetic acid–water (2:1:1 v/v) as solvent. The fractions containing aldosine **1** were evaporated to give a syrup. No characteristic absorption of visible light nor of UV light by aldosine **1** was observed. Both ^{1}H and ^{13}C NMR spectra of aldosine **1** were extremely complicated because of the presence of a great variety of structural types involving stereochemical variations (containing four asymmetric carbons), and of the tautomeric interconversion between imine and enamine products with probably very different chemical and physical properties in aq. solution. Treatment of connective tissue with hot alkali, which is a conventional method of preparing elastin, decreased the yield of aldosine **1**. An aldol crosslink might be destroyed by this treatment.

Synthesis and Structure Assignment of Anabasine-6′-carboxylic Acid **2**.—In order to synthesize compound **2**, the syrup containing aldosine **1** was diluted with water and then charged on a reaction column packed with activated charcoal which was supported with iron(III). Oxidative decarboxylation of compound **1** by activated charcoal supported with Fe^{3+} at room temperature produced compound **2** in theoretical yield, and the product **2** was fractionated subsequently from water, followed by elution with 50% aq. methanol. Purification of compound **2** by preparative HPLC on an ODS column with 0.1 mol dm^{-3} phosphate buffer–acetonitrile (5:1, v/v) containing 20 mmol dm^{-3} sodium dodecyl sulfate (SDS) as an ion pair at pH 4.0 in ~ 0.2% yield based on elastin by dry weight.

Although oxidative decarboxylation of aldosine **1** took place gradually during storage in air at room temperature, treatment with *N*-bromosuccinimide (NBS) at 90 °C for 10 min or with activated charcoal in water afforded compound **2**, although in far from quantitative yield. Interestingly, compound **2** was found to be formed from aldosine **1** by treatment with Fe^{3+}/activated charcoal in satisfactory yield.

UV Spectroscopy of compound **2** exhibited an absorption maximum at 260 nm (ε_{max} 5500) in 0.1 mol dm^{-3} HCl and at 260 nm (ε_{max} 3110) in 0.1 mol dm^{-3} NaOH, similar to that of anabasine (260 nm in both acidic and alkaline medium). The absorptions are characteristic of such 3-substituted pyridines as β-picoline (3-methylpyridine).

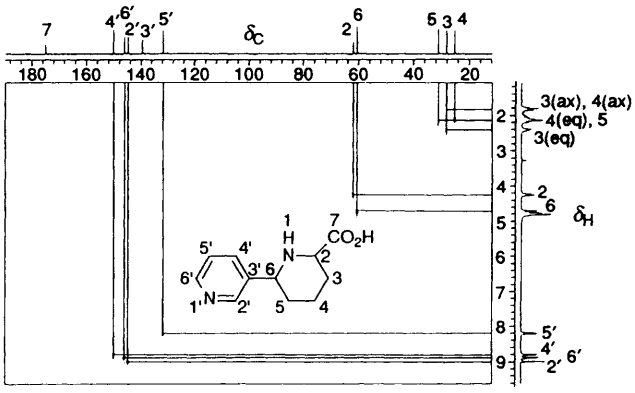


Fig. 1 ^{13}C–^{1}H COSY spectrum of anabasine-6′-carboxylic acid **2** in D_2O

The positive-ion FAB mass spectrum of compound **2** gave a relative molecular mass of 206 ($M + H^+$; 207, consistent with an elemental composition of $C_{11}H_{14}N_2O_2$). The NMR spectra of compound **2** are summarized in Table 1; assignments were confirmed by ^{1}H–^{1}H and ^{13}C–^{1}H correlated spectral data and by distortionless enhancement by polarization transfer (DEPT) data. The ^{1}H NMR spectrum of compound **2** contains a four-proton set (2′-H, 4′-, 5′- and 6′-H) for a 3-substituted pyridine (Fig. 1 and Table 1). In the ^{13}C NMR spectrum, the signals correspond to one carboxy group (δ_C 173.47), five pyridine ring carbons (δ_C 148.98, 145.02, 143.67, 138.52 and 130.89), one α-carbon to the pyridine (δ_C 61.43), one α-carbon to the imino acid group (δ_C 59.94), and three carbons not α to a functional group (δ_C 30.57, 27.64 and 24.73). ^{1}H–^{1}H Correlated spectroscopy and nuclear Overhauser effect (NOE) difference spectroscopy confirmed the proton assignments and established the chemical-shift values for axial and equatorial protons of compound **2**. In NOE experiments (Fig. 2), irradiation of 6-H (δ 4.70) resulted in enhancement of the signals attributable to 2′-H (δ 8.96) and 2-H (δ 4.23). These observations indicate that the pipecolic acid ring adopts a chair conformation and that the configuration of compound **2** can be specified as (2*S*, 6*R*) or (2*R*,6*S*). As compound **2** would be synthesized from L-lysine, though racemization could occur in the hydrolysis of elastin, the configuration should be (2*S*,6*R*). The FAB mass spectrum of the methyl ester derivative **4** of compound **2** gave a relative molecular mass of 220 ($M + H^+$; 221, consistent with an elemental composition of $C_{12}H_{16}N_2O_2$). This indicates that compound **2** has one carboxylic acid group. Catalytic reduction of compound **2** with palladium black afforded two compounds, 6-(piperidin-3-yl)pipecolic acid **5** and α-aminopiperidine-3-hexanoic acid **6**. An amine which is so substituted as to activate the alkylamino group of pipecolinic acid might be cleaved by hydrogen prior to saturation of the double bond which carries the activating effect. Decarboxylation of compound **2** by heating in paraffin oil at 250 °C for 20 min afforded anabasine **7** in detectable yield.

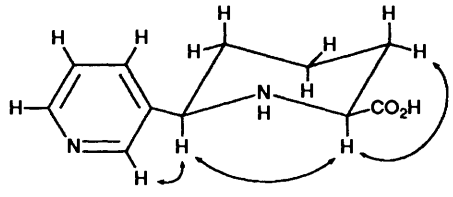


Fig. 2 Three-dimensional representation of anabasine-6′-carboxylic acid **2** showing selected NOE interactions

Pyridine compound **2** was named anabasine-6′-carboxylic acid because it is a carboxylic acid derivative of 2-(piperidin-3-yl)pyridine, anabasine,[7,8] which is one of the alkaloids in the

tobacco plant. Anabasine is used as an insecticide, has acute and subacute toxicity, and, in humans, causes increased salivation, vertigo, confusion, disturbed vision and hearing, and other cholinergic effects. There is a similarity in the structures of acetylcholine and such alkaloids as nicotine, anabasine, lobeline and compound **2**.

Experimental

General.—The solvents used for analytical HPLC were of HPLC grade, and of reagent grade for preparative HPLC, all being purchased from Nacalai Tesque (Kyoto, Japan). Activated charcoal (60–150 mesh) for column chromatography was obtained from Nacalai Tesque, and silica gel (Art. 7734) for column chromatography from Merck (Germany). Anabasine **7** was purchased from Sigma (USA). Analytical HPLC was performed on a reversed-phase Superspher RP-18 column (125 × 4 mm; Merck, Germany). The eluent was 0.1 mol dm^{-3} phosphate buffer–acetonitrile (5:1, v/v) containing 20 mmol dm^{-3} sodium dodecyl sulfate at pH 4.0. The flow rate was 1.0 cm^3/min. The HPLC system consisted of an LC-6A-pump, an SPD-6AV UV–VIS spectrophotometric detector, and a C-R5A data station (Shimadzu, Kyoto, Japan). The absorbance was monitored at 260 nm. TLC was conducted by using precoated Kieselgel 60 on an aluminium sheet (Merck, Art. 5553). The chromatogram was developed by ethyl acetate–acetic acid–water (2:1:1, v/v) as the solvent, and each spot on the plate was detected by spraying with 0.2% ninhydrin in 90% ethanol and then heating at 100 °C for 10 min.

Optical rotations were measured on a DIP-4 digital polarimeter (Japan Spectroscopic, Tokyo, Japan), and $[\alpha]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. The UV spectrum was obtained with a UV2100S spectrophotometer (Shimadzu, Kyoto, Japan), samples being dissolved in both 0.1 mol dm^{-3} HCl and 0.1 mol dm^{-3} NaOH. Mass spectrometric analysis was performed on a JMS HX-105 mass spectrometer (Japan Spectroscopic, Tokyo, Japan). Ions were generated by fast-atom bombardment (FAB), using a xenon primary beam of 70 eV energy. The sample was applied to a matrix consisting of a 1:1 mixture of water–glycerol. ^1H and ^{13}C NMR spectra were recorded at ambient temperature on a JNM GSX-400 spectrometer (Japan Spectroscopic, Tokyo, Japan), which was locked on for the D_2O solvent. *J*-Values are given in Hz.

Preparation of Elastin Hydrolysate.—The elastin hydrolysate of bovine ligamentum nuchae was prepared as follows. Freshly prepared cow's neck ligament was cut into small segments and washed with saline. After centrifugation, the precipitates were delipidated with chloroform–methanol (2:1, v/v). The delipidated sample (crude elastin) was dried over silica gel *in vacuo* and then hydrolysed in 6 mol dm^{-3} HCl (100 g of protein/dm^{-3} of acid) for 48 h at 110 °C in the conventional manner. The hydrolysate was evaporated to dryness under reduced pressure at 40 °C and subsequently stored at ~ −20 °C.

Aldosine **1**.—An acid hydrolysate of elastin (30 g, dry weight) was charged onto a large-scale borohydride-reduced charcoal column (65 × 200 mm). The major lysine-derived crosslinking amino acids containing aldosine **1** were fractionated from water (1500 cm^3) followed by elution with 50% aq. methanol (2 dm^3). The solvent was evaporated off to give a syrup, which was charged onto a preparative silica gel column (65 × 250 mm) for fractionation with ethyl acetate–acetic acid–water (2.5:1:1, v/v) as solvent. Fractions (200 cm^3) were collected. Aldosine, eluted just after arginine, gave a characteristically faint ninhydrin-positive (yellowish green) spot with an R_f-value of 0.16 (0.24 for arginine) on silica gel TLC. The fractions containing aldosine **1** were pooled (2400–3400 cm^3) and evaporated to give a syrup. This syrup was then charged onto a preparative HPLC ODS column (24 × 360 mm; RQ 2, Mitokagaku, Japan) with 10% aq. methanol as solvent. The flow rate was 4.0 cm^3/min and fractions (12 cm^3) were collected. Fractions 11–18 were pooled and evaporated to give a syrup, which was then charged onto a preparative, normal-phase HPLC column with ethyl acetate–acetic acid–water (2:1:1, v/v) as solvent. The fractions containing aldosine **1** were collected (360–456 cm^3). Aldosine is an amorphous solid that is soluble in aqueous solvents and in methanol but not in diethyl ether or chloroform; attempts to induce crystallization were unsuccessful (yield 105 mg); $\delta(D_2O)$ 3.9–4.2 [2 H, m, 2 × $CHCO_2D(ND)$], 3.62 and 3.28 (1 H, m, N=CH, ax, eq), 2.99 (1 H, td, NDCH <) and 1.45–2.51 [11 H, m, 5 × CH_2 + $ND(CO_2D)CHCH_2CH_2$]; *m/z* (FAB) 257 ($M + H^+$).

Preparation of Reaction Column for Oxidative Decarboxylation.—To synthesize compound **2** from aldosine **1**, Fe^{3+}/activated charcoal was used as the reagent for oxidative decarboxylation. An activated charcoal column (65 × 180 mm) was treated with excess of aq. 10% iron(III) sulfate. The column was washed successively with excess of water, 50% aq. methanol, methanol and then these solvents in the reverse order.

Anabasine-6′-carboxylic Acid **2**.—Anabasine-6′-carboxylic acid **2** was prepared from aldosine **1**. A solution of aldosine **1** in distilled water was applied to the Fe^{3+}/activated charcoal reaction column. Anabasine-6′-carboxylic acid **2** was fractionated from water (1500 cm^3) followed by elution with 50% aq. methanol (2000 cm^3). The 50% methanol fraction was evaporated to dryness under reduced pressure and the residue was loaded onto a preparative HPLC ODS column with a solvent of 0.1 mol dm^{-3} phosphate buffer–acetonitrile (5:1, v/v) containing 20 mmol dm^{-3} SDS (pH 4.0). To remove SDS from the fraction containing title compound **2**, potassium chloride (10 mol equiv.) was added and the fraction was left overnight in a refrigerator. The resulting potassium dodecyl sulfate was removed by filtration. The filtrate was evaporated to dryness under reduced pressure and the title compound **2** was extracted with methanol (3 × 30 cm^3). The solvent was evaporated off under reduced pressure and the resulting residue was chromatographed on a silica gel preparative HPLC column with a solvent mixture of ethyl acetate–acetic acid–water (2.5:1:1, v/v). Confirmation was achieved by reversed-phase HPLC. Fractions corresponding to a t_R-value of 11 min (phenylalanine t_R 9.5 min; desmosine t_R 22.5 min) on HPLC were concentrated to give *title compound* **2** as a pale yellow syrup (65 mg), $[\alpha]_D^{22}$ −3.1 (*c* 0.267, MeOH), −6.2 (*c* 0.267, water); λ_{max}(0.1 mol dm^{-3} HCl)/nm 260 (ε 5500); λ_{max}(0.1 mol dm^{-3} NaOH)/nm 260 (ε 3110); ν_{max}(film)/cm^{-1} 3350, 2930, 1735, 1640, 1560, 1420, 1220, 1100 and 1010; ^1H and ^{13}C NMR, see Fig. 1 and Table 1 (Found: $M + H^+$, 207.1134. $C_{11}H_{14}N_2O_2$ + H requires *m/z*, 207.1133).

Methyl Anabasine-6′-carboxylate **4**.—Anabasine-6′-carboxylic acid **2** (10 mg) was esterified by methanol in the presence of 5% hydrogen chloride at 80 °C for 6 h in flame-sealed Pyrex tubes; λ_{max}(0.1 mol dm^{-3} HCl)/nm 260 (ε 5500); λ_{max}(0.1 mol dm^{-3} NaOH)/nm 260 (ε 3110); *m/z* (FAB) 221 ($M + H^+$).

Preparation of 6-(Piperidin-3-yl)pipecolic Acid **5** *and 2-Amino-6-(piperidin-3-yl)hexanoic Acid* **6**.—Anabasine-6′-carboxylic acid **2** (50 mg) was reduced with Pd black (10 mg) in water (20 cm^3) under hydrogen for 12 h at room temperature. The resulting mixture of 6-(piperidin-3-yl)pipecolic acid **5** and 2-amino-6-(piperidin-3-yl)hexanoic acid **6** was separated on a silica gel preparative HPLC column with ethyl acetate–acetic acid–water

(2.5:1:1, v/v). Confirmation was achieved by silica gel TLC. Compounds **5** and **6** were chromatographed by TLC with ethyl acetate–acetic acid–water as a faint ninhydrin-positive (yellow) spot (R_f 0.13) (**5**) and ninhydrin-positive (purple) spot (R_f 0.17) (**6**), respectively.

6-(*Piperidin-3-yl*)*pipecolic acid* **5**, mixed crystals of isomers (35.2 mg); m.p. darkens at 250 °C, 260–265 °C (decomp.) (from MeOH); $[\alpha]_D^{22}$ −2.7 (*c* 0.352, MeOH), −5.4 (*c* 0.352, water); ν_{max}(KBr)/cm^{-1} 3350, 3130, 2930, 2800, 1740, 1620, 1400 and 1200; δ 1.48 and 1.63 (each 2 H, m), 1.72, 2.17 and 2.28 (each 1 H, m), 2.0, (4 H, m), 2.91 and 3.25 (each 2 H, m), 3.42 (1 H, d, *J* 12), 3.53 (1 H, m) and 3.98 (1 H, t, *J* 12) (Found: M + H$^+$, 213.1604. $C_{11}H_{20}N_2O_2$ + H requires *m/z* 213.1603).

2-*Amino*-6-(*piperidin-3-yl*)*hexanoic acid* **6**, mixed crystals of isomers (2.4 mg); m.p. darkens at 220 °C, 230–240 °C (decomp.) (from MeOH); ν_{max}(KBr)/cm^{-1} 3410, 3100, 3030, 2935, 2800, 1740, 1625, 1510, 1400 and 1210; δ 1.15 and 1.30 (each 1 H, m), 1.4 (5 H, m), 1.7 (2 H, m), 1.9 (5 H, m), 2.61 (1 H, t, *J* 12), 2.86 (1 H, t, *J* 12), 3.32 (2 H, d, *J* 12) and 3.98 (1 H, t, *J* 6) (Found: M + H$^+$, 215.1760. $C_{11}H_{22}N_2O_2$ + H requires *m/z*, 215.1759).

Anabasine **7**.—Anabasine-6′-carboxylic acid **2** was heated with paraffin oil at 250 °C for 20 min. Anabasine **7** and compound **2** were separated by TLC with diethyl ether–methanol–25% aq. ammonia (60:50:1, v/v). Anabasine **7** thus synthesized was detected by Dragendorff's reagent and identified by comparison with authentic material. R_f-Values: anabasine **7** 0.13, anabasine-6′-carboxylic acid **2** 0.37.

Acknowledgements

We thank Dr. M. Murata, Mrs. Y. Murata and Mrs. Y. Sugiyama for helpful discussions.

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Paper 3/00409K
Received 22*nd January* 1993
Accepted 8*th February* 1993