Phytochemistry 70 (2009) 100–104

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Azetidine-2-carboxylic acid in the food chain

Edward Rubenstein [a,*], Theresa McLaughlin [b,1], Richard C. Winant [c], Agustin Sanchez [c,2], Michael Eckart [c,3], Karolina M. Krasinska [b,1], Allis Chien [b,4]

[a] Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305, United States
[b] The Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University Mass Spectrometry, United States
[c] Beckman Center, Stanford Medical Center, Stanford, CA 94305, United States

ARTICLE INFO

Article history:
Received 6 February 2007
Received in revised form 8 October 2008
Available online 26 December 2008

Keyword:
Sugar beet
Beta vulgaris
Chenopodiaceae
Azetidine-2-carboxylic acid (Aze)
Multiple sclerosis
p53

ABSTRACT

Azetidine-2-carboxylic acid (Aze) **1** is a non-protein amino acid present in sugar beets and in table beets (*Beta vulgaris*). It is readily misincorporated into proteins in place of proline **2** in many species, including humans, and causes numerous toxic effects as well as congenital malformations. Its role in the pathogenesis of disease in humans has remained unexplored. Sugar beet agriculture, especially in the Northern Hemisphere, has become widespread during the past 150 years, and now accounts for nearly 30% of the world's supply of sucrose. Sugar beet byproducts are also used as a dietary supplement for livestock. Therefore, this study was undertaken as an initial survey to identify Aze-containing links in the food chain. Herein, we report the presence of Aze **1** in three sugar beet byproducts that are fed to farm animals: sugar beet molasses, shredded sugar beet pulp, and pelleted sugar beet pulp.

1. Introduction

Azetidine-2-carboxylic acid (Aze) **1** (Fig. 1) is a plant non-protein amino acid identical to proline **2**, except that the ring of Aze **1** has four members and the ring of proline **2** has five (Couty and Evano, 2006). Fowden discovered Aze **1** in lilies, poincianas, and sugar beets, and subsequently showed that it deters the growth of competing vegetation and poisons predators as a result of its misincorporation into proteins in place of proline **2** (Fowden, 1956). The tRNA mechanism of the plants that accumulate Aze **1** discriminates between proline **2** and Aze **1**, and therefore these plants avoid autotoxicity.

The noxious effects of Aze **1** have been shown to result in a wide range of toxic and teratogenic disorders in various species, including chicks, ducks, hamsters, mice, and rabbits (Rubenstein, 2000; Rubenstein et al., 2006). Misassembly of human proteins, such as collagen and hemoglobin, has been reported as well (Rubenstein, 2000).

Whether Aze **1** causes diseases in humans is not currently known. Recently, Aze **1** has been found in table beets, a staple component of the human diet in some regions (Rubenstein et al., 2006). The concentration of free Aze **1** in table beets varied from 1% to 5% of that of free proline **2** (Rubenstein et al., 2006).

Sugar beets are the source of about 30% of the world's supply of sucrose. Byproducts of sugar beets are fed to meat and dairy livestock, and in this way their constituents could enter food consumed by humans and other animals. (Harland et al., 2006) Because of the well-established noxious effects of Aze **1**, we undertook this initial survey to identify Aze-containing links in the sugar beet food chain.

Here we present the results of liquid chromatography–mass spectrometry of three sugar beet byproducts: shredded dried sugar beet pulp, pelleted dried sugar beet pulp, and sugar beet molasses.

2. Results and discussion

Aze **1** was found in sugar beet molasses and in two different varieties of sugar beet dried pulp (Figs. 2–5).

The concentrations of proline **2**, Aze **1**, and threonine **3** are summarized in Table 1.

In addition to the two target compounds, proline **2** and Aze **1**, it was necessary to monitor threonine **3** during the LC–MS/MS assay. The amino acid threonine **3** is present in significant quantities in sugar beets. During electrospray ionization, threonine **3** can

* Corresponding author. Address: General Internal Medicine, 251 Campus Drive, MSOB X216, Stanford Medical Center, Stanford, CA 94305, United States. Tel.: +1 650 723 8287; fax: +1 650 723 8596.
E-mail addresses: exr@stanford.edu (E. Rubenstein), tmcl@stanford.edu (T. McLaughlin), asanchez@cmgm.stanford.edu (A. Sanchez), meckart@stanford.edu (M. Eckart), krasin@stanford.edu (K.M. Krasinska), allis@stanford.edu (A. Chien).
[1] Tel.: +1 650 725 9769; fax: +1 650 723 4817.
[2] Tel.: +1 650 3328; fax: +1 650 725 0766.
[3] Tel.: +1 650 723 1907; fax: +1 650 725 0766.
[4] Tel.: +1 650 723 0718; fax: +1 650 7243 4817.

0031-9422/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2008.11.007

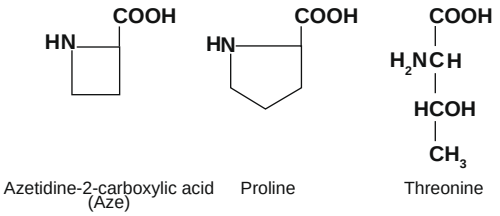

Fig. 1. The structures of azetidine-2-carboxylic acid **1**, proline **2**, and threonine **3**.

undergo a neutral loss of water in the mass spectrometer source, forming a pseudomolecular ion at *m*/*z* 102 with the same molecular composition. Therefore it was important to demonstrate that Aze **1** and threonine **3** were chromatographically separated, as shown in Fig. 2. Distinct peaks were observed for Aze **1** and threonine **3**, indicating that Aze **1** was separated from the threonine **3** dehydration artifact. This result confirms the findings previously reported (Rubenstein et al., 2006).

The misincorporation of Aze **1** in proteins in place of proline **2** may be pertinent to disease in humans, in livestock, in domesticated animals fed rations containing sugar beet byproducts, and perhaps in free-living animals that forage on sugar beets. Many investigations have established the fact that Aze **1** produces deleterious effects; however, data based on toxicologic studies are needed to define such parameters as dose, route of administration, duration, and age at time of exposure.

For instance, the incubation of anemic rabbit reticulocytes with 1, 5, and 10 mM Aze **1** has been shown to reduce the incorporation of radiolabled proline **2** into hemoglobin by 25%, 58%, and 72%, respectively (Baum et al., 1975). However, the results of such studies cannot be translated into quantitative information relevant to toxicology.

Another example is that of dysmorphogenesis of the murine inner ear caused by exposure of isolated otic explants from 10.5-day-old through 14-day-old mice embryos to a nutrient medium containing Aze **1** in concentrations of 75, 150, and 300 μg/ml. Aze **1** disrupted morphogenesis in mice after 10.5–13 days of gestation, but not in those after 14 days of gestation. Thus, embryonic age at the time of exposure appears to be a critical factor in the expression of teratogenesis at various dose levels (Van de Water and Galinovic-Schwartz, 1986).

There are a number of examples of Aze-induced protein dysfunction that may be relevant to disease in humans. The site-directed replacement of proline **2** by Aze **1** in an ion channel protein has been shown to impair the regulation of aperture size and thus channel function (Lummis et al., 2005).

Prolyl residues play a key role in the hypoxia-inducible factor (HIF) (Schofield and Ratcliffe, 2004). The replacement of proline **2** by Aze **1** in the molecular complex has been shown to impair the function of the HIF mechanism (Li et al., 2004).

Another example is that of collagen, in which proline **2** comprises about 15% of the amino acids (Okuyama et al., 1981). Substitution of Aze **1** for proline **2** in collagen alters mechanical properties and destabilizes the collagen triple helix (Zagari et al., 1990). Normal human skin fibroblasts grown in culture in the presence of Aze **1** produce markedly abnormal collagen fibrils that fail to fold properly into a triple-helical conformation (Tan et al., 1983). In another study, the topical application of Aze **1** (0.2 mg in 0.1 ml of water) to split-thickness skin wounds in young pigs decreased the rate of epithelialization and diminished the levels of hydroxyproline in the dermis (Alvarez et al., 1982).

Proteins that may be especially vulnerable to the deleterious effects of the misincorporation of Aze **1** are those bearing proline-rich domains that are critically involved in structure or function.

For instance, the tumor suppressor protein p53 integrates responses to oncogene activation, to DNA damage, and to a wide range of noxious cell stresses. Under such circumstances p53 arrests the cell cycle to allow increased time for DNA repair, and when damage is severe p53 initiates apoptosis. A highly conserved

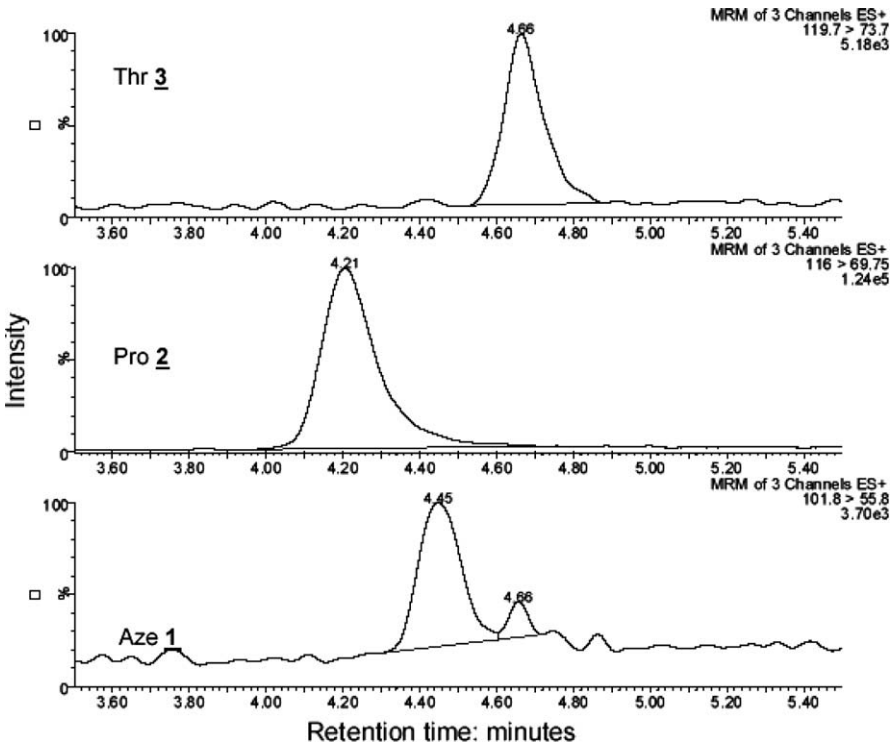

Fig. 2. Chromatograms of standards for threonine **3**, proline **2**, and Aze **1** at equal concentrations. Aze **1** elutes at 4.45 mins, and threonine **3** elutes at 4.66 min. The threonine **3** dehydration artifact is visible as a peak in the Aze **1** chromatogram at 4.66 min.

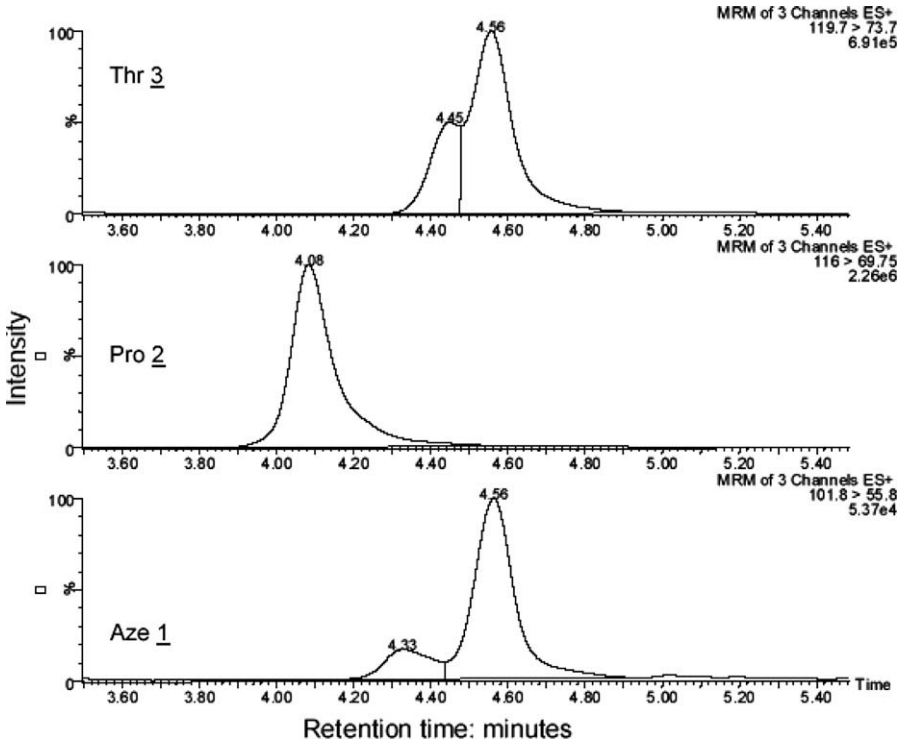

Fig. 3. Chromatogram showing the presence of Aze **1** in sugar beet molasses. The concentration of threonine **3** in the sample is significantly greater than that of Aze **1**. For this reason the chromatographic peak for the dehydration artifact is larger than the peak of Aze **1**.

proline-rich domain in p53, which mediates the apoptotic response, presents numerous opportunities for misincorporation. Proline **2** residues account for seven of the 16 amino acids in this sequence (Aylon and Oren, 2007; Bergamaschi et al., 2006; Levine et al., 1991; Hollstein et al., 1991). Corruption of the apoptosis-initiating portion of the molecule could allow a cell that had suffered a malignancy producing mutation to escape destruction and to initiate a neoplastic clone (Nelson et al., 2005).

Myelin basic protein, a long-lived molecule, is another example of a protein in which there is a critical proline-rich sequence that

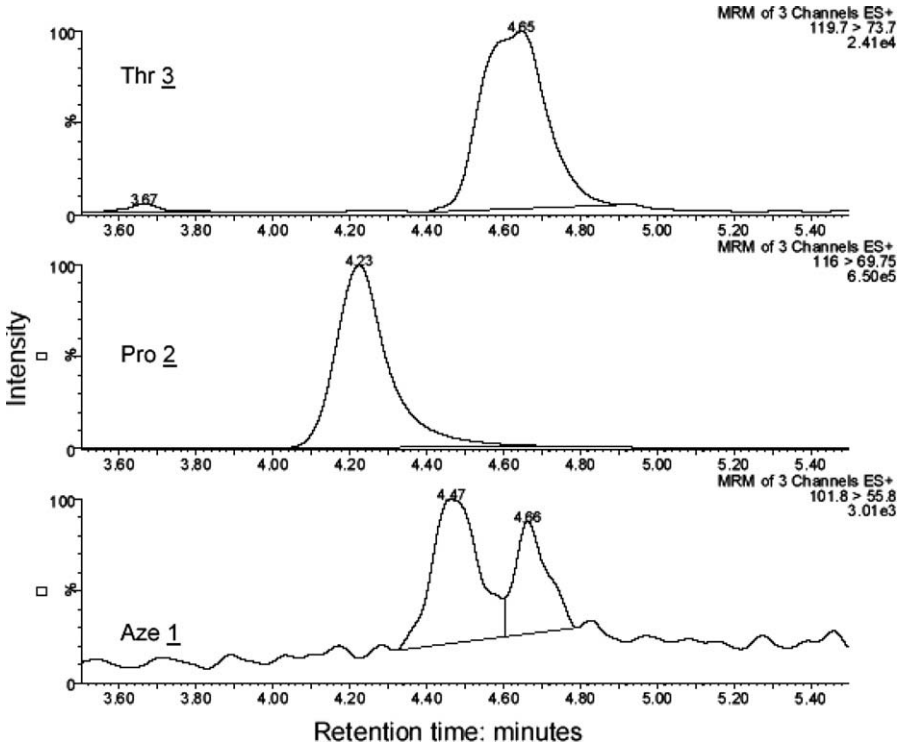

Fig. 4. Chromatogram showing the presence of Aze **1** in shredded sugar beet pulp.

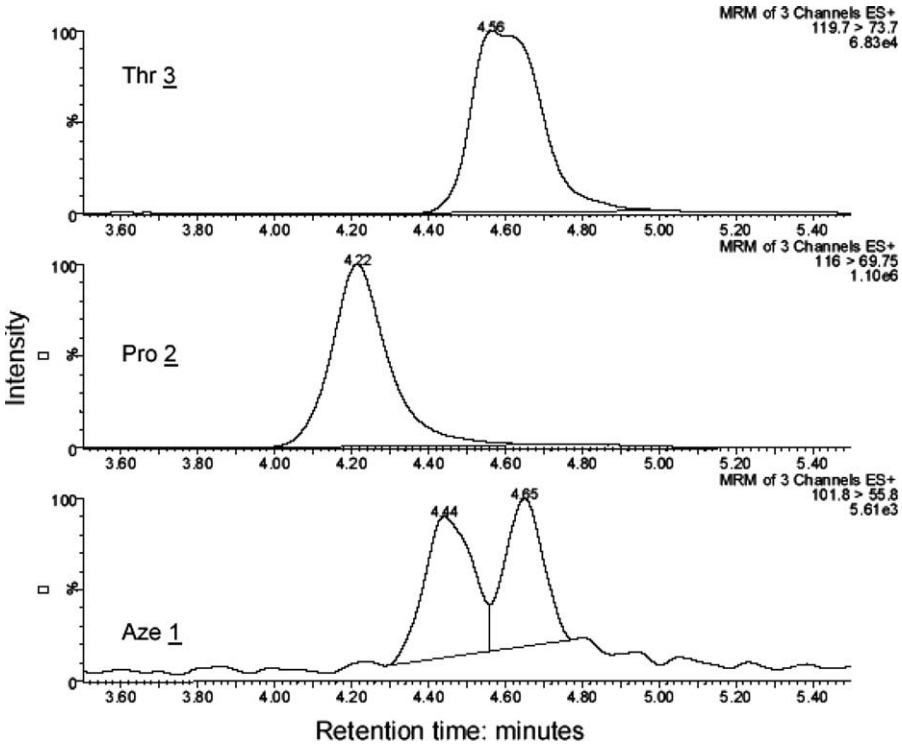

Fig. 5. Chromatogram showing the presence of Aze **1** in pelleted sugar beet pulp.

Table 1
Detected concentrations of proline **2**. Aze **1** and threonine **3** in sugar beet by products

Product	Proline **2** (mg/100 g)	Aze **1** (mg/100 g)	Threonine **3** (mg/100 g)	Pro/Aze ratio
Molasses	350	0.340	22	1.03e3
Shredded beet pulp	9.1	0.058	3.2	1.63e2
Pelleted beet pulp	2.7	0.021	1.5	1.28e2

may be vulnerable to the effects of Aze **1** misincorporation. This protein is a principal component of the sheath surrounding myelinated axons of the central nervous system. Numerous lines of evidence indicate that the characteristic plaques of multiple sclerosis arise in these axon sheaths and that there is damage and subsequent loss of myelin basic protein within these lesions (Boggs, 2006).

A consensual epitope of myelin basic protein, residues 89–101, includes a hexapeptide sequence containing four prolines: Pro-ArgThrProProPro. The triproline chain, which is highly conserved, has been regarded as a rigid keystone of the molecule (Ridsdale et al., 1997). Another string of amino acids (residues 85–96), an α-helix bounded by prolyls, has also been identified as antigenic (Bates et al., 2004). Substitution by Aze **1** for one or more prolines **2** in these sequences could cause significant structural, functional, and immunogenic alterations of the protein (Jhon and Kang, 2007).

3. Concluding remarks

We present data indicating that azetidine-2-carboxylic acid (Aze) **1** is present in sugar beet byproducts that are used in the feeding of dairy and other livestock. These findings suggest that the non-protein amino acid may be transferred from sugar beets into other foods. The concentrations of Aze **1** in these byproducts are far lower than those in table beets.

The lack of detailed toxicologic data and the need for more direct evidence about the damaging effects of the misincorporation of Aze **1** on specific proteins are strong reasons for exercising restraint regarding the role of Aze **1** in disease in humans. We caution against premature conclusions about dietary modifications.

4. Experimental

Three forms of processed sugar beets (*Beta vulgaris*) were analyzed. Sugar beet molasses was obtained from the Spreckles Sugar® Company, Mendota, CA. Shredded beet pulp was a product of Spreckles Agriculture Department, Mendota, CA. Pelleted beet pulp was a product of the Nutrena Company, Minneapolis, MN.

Sugar beet molasses (200 mg) were diluted with 2 mM ammonium formate (1 ml) and vortexed to mix. A 1:100 dilution of this solution in 30 mM NH_4OAc:CH_3CN (1:3, v/v) was prepared for LC–MS analysis.

Sugar beet pulp: Sugar beet shredded pulp (2 g dry weight) was added to doubly distilled H_2O (50 ml); the suspension was then placed in a boiling water bath for two hours. Sugar beet pelleted pulp was treated identically. Following storage at 8 °C overnight, the extracts were individually filtered through #1 weight filter paper. About 15 ml of each liquid were recovered per sample. The liquid from each sample was divided into two Falcon tubes and dried in a Speed Vac. After drying, approximately 1 ml of clear, viscous material remained in each tube.

One tube from each sample was analyzed. The material in the tube was resuspended in H_2O (5 ml) using vortexing, sonication and stirring with a spatula to solubilize the material. Aliquots were diluted 1:10 and 1:100 (v/v) with mobile phase buffer and injected into the LC–MS. Standard and sample chromatograms are shown in Figs. 2–5.

The samples were analyzed by LC–MS/MS on an Agilent 1100 HPLC system and Waters Quattro Premier MS with electrospray ionization source. The HPLC method is adapted from Bajad et al. (2006). A 150 × 2.0 mm 5 μm Luna NH_2 column from Phenomenex S/N 4002994-3 was used with a flow rate of 300 μl/min. The meth-

od conditions were initially a 1:3 ratio (v/v) 25% A (20 mM NH_4OAc plus 20 mM NH_4OH in H_2O, pH 9)/75% B (CH_3CN), this being ramped to A:B (6:4, v/v) in 5 min, and then returned to A:B (1:3, v/v) at 6 min, which was held for an additional 4 min.

For proline **2** analysis, the method monitored the transition from 116 → 70, and the calibration curve spanned 0.18–2.9 ng on column. For threonine **3** analysis the method monitored the transition from 120 → 74, and the calibration curve spanned 0.037–0.6 ng on column. For Aze **1** analysis the method monitored the transition from 102 → 56, and the calibration curve spanned 0.16–0.25 ng on column.

L-Azetidine-2-carboxylic acid **1** Aldrich, A0760, L-proline **2** (Fluka, 81709), and L-threonine **3** (Fluka, 89179) were obtained from the manufacturers and used without further purification.

Acknowledgment

This work was supported in part by the Technology Innovation Fund, Beckman Center, at the Stanford University Medical Center, Stanford, CA.

References

Alvarez, O.M., Mertz, P.M., Eaglstein, W.H., 1982. The effect of the proline analogue l-azetidine-2-carboxylic acid (LACA) on epidermal and dermal wound repair. Plast. Reconstr. Surg. 69, 284–289.

Aylon, Y., Oren, M., 2007. Living with p53, dying of p53. Cell 130, 597–600.

Bajad, S.U., Lu, W., Kimball, E.H., Yuan, J., Peterson, C., Rabinowitz, J.D., 2006. Separation and quantitation of water soluble cellular metabolites by hydrophilic interaction chromatography–tandem mass spectrometry. J. Chromatogr. A 1125, 76–88.

Bates, I.R., Feix, J.B., Boggs, J.M., Harauz, G., 2004. An immunodominant epitope of myelin basic protein is an amphipathic α-helix. J. Biol. Chem. 279, 5757–5764.

Baum, B.J., Johnson, L.S., Franzblau, C., Troxler, R.F., 1975. Incorporation of L-azetidine-2-carboxylic acid into hemoglobin in rabbit reticulocytes *in vitro*. J. Biol. Chem. 250, 1464–1471.

Bergamaschi, D., Samuels, Y., Sullivan, A., Zvelebil, M., Breyssens, H., Bisso, A., Del Sal, G., Syed, N., Smith, P., Gasco, M., Crook, T., Lu, X., 2006. IASPP preferentially binds p53 proline-rich region and modulates apoptotic function of codon 72-polymorphic p53. Nat. Genet. 38, 1133–1141.

Boggs, J.M., 2006. Myelin basic protein: a multifunctional protein. Cell Mol. Life Sci. 63, 1945–1961.

Couty, F., Evano, G., 2006. Azetidine-2-carboxylic acid. From lily of the valley to key pharmaceuticals, a jubilee review. Org. Prep. Proc. Int. 38, 427–466.

Fowden, L., 1956. Azetidine-2-carboxylic acid: a new cyclic amino acid occurring in plants. Biochem. J. 64, 323–332.

Harland, J.J., Jones, C.K., Hufford, C., 2006. Co-products. In: Draycott, A.P. (Ed.), Sugar Beet. Blackwell Publishing Ltd., pp. 443–463.

Hollstein, M., Sidransky, E., Vogelstein, B., Harris, C.C., 1991. P53 mutations in human cancers. Science 253, 49–53.

Jhon, J.S., Kang, Y.K., 2007. Conformational preferences of proline analogues with different ring size. J. Phys. Chem. B 111, 3496–3507.

Li, D., Hirsilä, M., Koivunen, P., Brenner, M.C., Xu, L., Yang, C., Kivirikko, K.I., Myllyharju, J., 2004. Many amino acid substitutions in hypoxia-inducible transcription factor (HIF)-1α-like peptide cause only minor changes in its hydroxylation by the HIF prolyl 4-hydroxylases. Substitution of 3,4 dehydroproline or azetidine-2-carboxylic acid for the proline leads to a high rate of uncoupled 2-oxoglutarate decarboxylation. J. Biol. Chem. 279, 55051–55059.

Levine, A.J., Momand, J., Finlay, C.A., 1991. The p53 tumour suppressor gene. Nature 351, 453–456.

Lummis, S.C., Beene, D.L., Lee, L.W., Lester, H.A., Broadhurst, R.W., Dougherty, D.A., 2005. *Cis–trans* isomerization at a proline opens the pore of a neurotransmitter-gated ion channel. Nature 438, 248–252.

Nelson, H.H., Wilkojmen, M., Marsit, C.J., Kelsey, K.T., 2005. TP53 mutation, allelism and survival in non-small cell lung cancer. Carcinogenesis 26, 1770–1773.

Okuyama, K., Okuyama, K., Arnott, S., Takayanagi, M., Kakudo, M., 1981. Crystal and molecular structure of a collagen-like polypeptide (Pro-Pro-Gly) 10. J. Mol. Biol. 152, 427–443.

Ridsdale, R.A., Beniac, D.R., Tompkins, T.A., Moscarello, M.A., Harauz, G., 1997. Three-dimensional structure of myelin basic protein. Molecular modeling and considerations of predicted structures in multiple sclerosis. J. Biol. Chem. 272, 4269–4275.

Rubenstein, E., 2000. Biologic effects and clinical disorders caused by nonprotein amino acids. Medicine 79, 80–89.

Rubenstein, E., Zhou, H., Krasinska, K.M., Chien, A., Becker, C.H., 2006. Azetidine-2-carboxylic acid in garden beets (*Beta vulgaris*). Phytochemistry 67, 898–903.

Schofield, C.J., Ratcliffe, P.J., 2004. Oxygen sensing by HIF hydroxylases. Nat. Rev. Mol. Cell Biol. 5, 343–354.

Tan, E.M., Ryhanen, I., Uitto, J., 1983. Proline analogues inhibit human skin fibroblast growth and collagen production in culture. J. Invest. Dermatol. 80, 261–267.

Van de Water, T.R., Galinovic-Schwartz, V., 1986. Dysmorphogenesis of the inner ear: the disruption of extracellular matrix (ECM) formation by an L-proline analog in otic explants. J. Craniofac. Genet. Dev. Biol. 6, 113–129.

Zagari, A., Enmity, G., Scheraga, H.A., 1990. The effect of L-azetidine-2-carboxylic acid residue on protein conformation. I. Conformations of the residue and of dipeptides. Biopolymers 30, 951–959.