# $\Delta^3$-Piperideine Alkaloids from the Toxic Plant *Lobelia berlandieri*

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Three piperideine alkaloids were isolated from ether extracts of the poisonous weed *Lobelia berlandieri*. Purification was achieved by acid–base extraction and preparative liquid and gas chromatography. Analysis by mass spectroscopy and $^1H$ and $^{13}C$ NMR showed the compounds to be $\Delta^3$-*N*-methylpiperideine derivatives, substituted in the 2- and 6-positions to yield *N*-methyl-2,6-bis(2-hydroxybutyl)-$\Delta^3$-piperideine (**1**), *N*-methyl-2-(2-hydroxypropyl)-6-(2-hydroxybutyl)-$\Delta^3$-piperideine (**2**), or *N*-methyl-2-(2-oxobutyl)-6-(2-hydroxybutyl)-$\Delta^3$-piperideine (**3**).

*Lobelia berlandieri* is an annual plant that grows during spring months in southern Texas and northern Mexico. Sporadic but occasionally heavy losses of cattle due to ingestion of *L. berlandieri* have been reported over the years, the most recent occurrence being in 1979 (Hubert, 1983). Animals affected undergo prolonged periods of narcosis lasting up to 2 weeks but, if hand-fed and watered while comatose, can recover. The compound lobeline produces similar symptoms and was believed to be responsible (Dollahite and Allen, 1962). We found no lobeline in *L. berlandieri* extracts, but rather several related compounds.

## EXPERIMENTAL SECTION

**Isolation of Alkaloids.** Plant material was collected near Riviera, TX, in April 1979 and April 1981. Identification was confirmed by comparison with deposits in the herbarium, Department of Plants Sciences, Texas A&M University. In a typical procedure, 200 g of whole, air-dried *L. berlandieri* was ground in a Wiley mill, mixed with 200 g of sand and 200 mL of 10% $NH_3$ in $H_2O$, and stirred vigorously for 5 min with 500-mL portions of peroxide-free ether. The ether extraction was repeated twice. The combined decanted ether washes were extracted with 2 × 200 mL of 2% $H_2SO_4$. The water layers were quickly cooled to <5 °C, the pH was carefully adjusted to 8–8.5 with 10% $NH_3$ in $H_2O$, and the basic solution was immediately extracted with 2 × 500 mL of $CHCl_3$. This procedure is in general that of Borio and Kawese (1967). The $CHCl_3$ extract was dried over $Na_2SO_4$, evaporated on 5 mL of 60–80-mesh neutral alumina, and added to a 20 cm × 2 cm alumina in hexane column. The column was eluted successively with 100 mL of hexane and 500 mL of ether, and 50-mL fractions were collected. Separation was monitored by TLC on silica gel G-60 with $CHCl_3$–MeOH (7:3) as solvent using Dragandorff's reagent visualization. Fractions 5–7 and 8 and 9 contained compounds of interest and were subjected to further purification using preparative gas chromatography. Compounds 1 and **2** were found in LC fractions 8 and 9, and compound **3** was found in LC fractions 5–7.

**Instrumentation.** Gas chromatography was performed on a Tracor 550 GC with flame ionization detection modified for preparative use with an all-glass 105-0:1 continuously variable splitter system. A 1.83 m × 4 mm i.d. glass column packed with 3% OV101 on Chromosorb 750 was used, flow rate 60 mL/min of $N_2$, operated isothermally at 160 °C. Fractionated samples were collected in 30 cm × 1.3 mm o.d. glass capillary tubes at liquid nitrogen temperature by a Brownlee–Silverstein thermal gradient collector (Brownlee and Silverstein, 1968). NMR spectra were recorded on JEOL FX90Q, Varian XL200, or Bruker AM-500 Fourier transform spectrometers using $CDCl_3$ [$(CD_3)_2CO$ for 500 MHz] in 1.7- or 5-mm tubes. Mass spectra were recorded on a Hewlett-Packard 5992B quadrupole GC–MS using a 1.83 m × 2 mm i.d. 3% OV1 on Gas Chrom Q 80–100-mesh column [He flow rate 30 mL/min, temperature programmed 160–260 °C at 10 °C/min] or a Nicolet FT-MS 1000 GC–MS instrument using a 25 m × 0.3 mm i.d. vitreous silica BP1 column [12 lb/in$^2$ He head pressure, programmed 60 °C for 1 min and then 20 °C/min to 220 °C]. Infrared spectra were obtained from $CHCl_3$ solutions with a Perkin-Elmer model instrument.

**Table I. Mass Spectral Data of Compounds 1–3**

| compd 1 | | compd 2 | | compd 3 | |
|---|---|---|---|---|---|
| *m/z* | rel abund, % | *m/z* | rel abund, % | *m/z* | rel abund, % |
| 41 | 20 | 41 | 17 | 41 | 13 |
| 42 | 38 | 42 | 39 | 42 | 28 |
| 44 | 9 | 43 | 9.5 | 43 | 5 |
| 53 | 3.5 | 44 | 10 | 44 | 7 |
| 55 | 4.5 | 45 | 17 | 53 | 3 |
| 56 | 11 | 53 | 4 | 55 | 4.5 |
| 57 | 13 | 55 | 4 | 56 | 8 |
| 58 | 37 | 56 | 11 | 57 | 39 |
| 59 | 18 | 57 | 19 | 58 | 21 |
| 67 | 8 | 58 | 37 | 59 | 6 |
| 68 | 11 | 59 | 8.5 | 67 | 7 |
| 70 | 3.5 | 67 | 9.5 | 68 | 4 |
| 79 | 3.5 | 68 | 12 | 70 | 2.5 |
| 81 | 4 | 70 | 2 | 79 | 3.5 |
| 82 | 3.5 | 79 | 4 | 80 | 3 |
| 86 | 12 | 80 | 3 | 81 | 4 |
| 94 | 47 | 81 | 4.5 | 82 | 4 |
| 95 | 6 | 82 | 4 | 86 | 6.5 |
| 96 | 100 | 86 | 13 | 94 | 100 |
| 97 | 7 | 94 | 47 | 95 | 11 |
| 108 | 8.5 | 95 | 7 | 96 | 54 |
| 110 | 16 | 96 | 100 | 97 | 4 |
| 116 | 13 | 97 | 8.5 | 108 | 13 |
| 138 | 1 | 108 | 9 | 110 | 26 |
| 168 | 76 | 110 | 20 | 116 | 3 |
| 169 | 14 | 116 | 15 | 124 | 3.5 |
| 182 | 1 | 138 | 3 | 138 | 13 |
| 212 | 9.5 | 154 | 25 | 166 | 12 |
| 213 | 1.5 | 155 | 8.5 | 167 | 18 |
| 226 | 1.5 | 168 | 50 | 168 | 29 |
| 241 | 1 | 169 | 7.5 | 169 | 3 |
| | | 182 | 1.5 | 182 | 11 |
| | | 198 | 5 | 210 | 4.5 |
| | | 212 | 3 | 224 | 4 |
| | | 227 | 1 | 239 | 1 |

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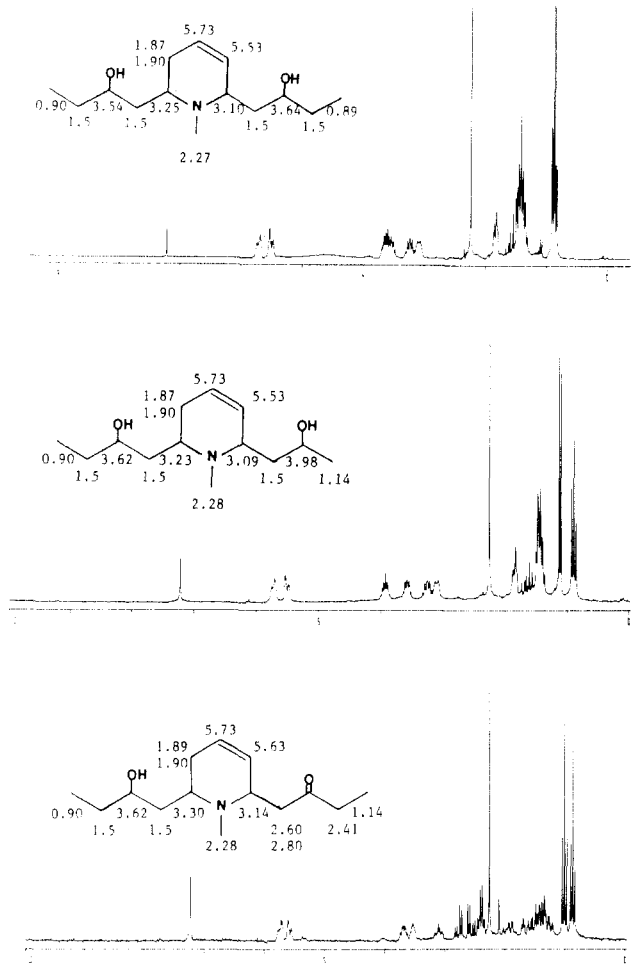



**Figure 1.** 200-MHz $^1H$ NMR spectra of compounds 1–3 with assignments.

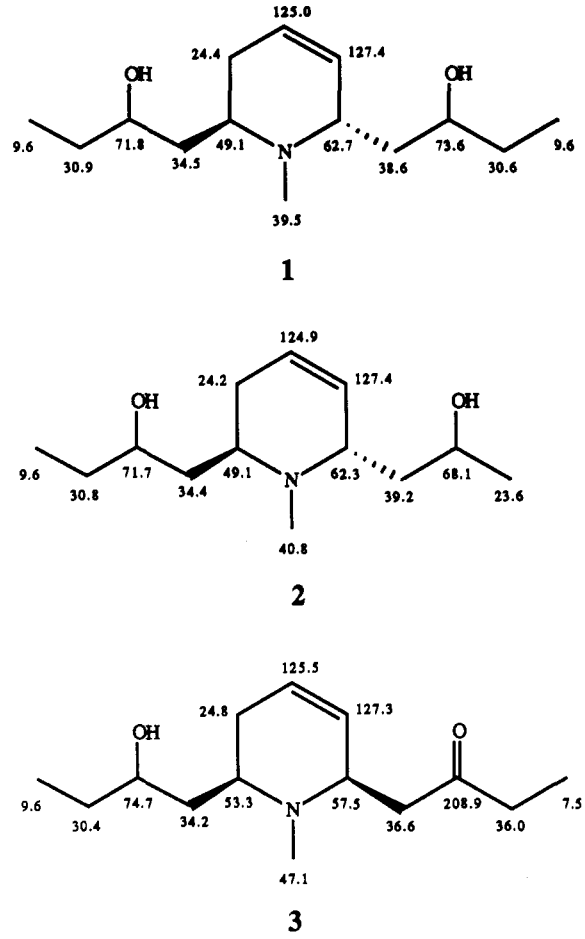



**Figure 2.** Compounds 1 [*N*-methyl-2,6-bis(2-hydroxybutyl)-$\Delta^3$-piperideine], 2, [*N*-methyl-2-(2-hydroxypropyl)-6-(2-hydroxybutyl)-$\Delta^3$-piperideine], and 3 [*N*-methyl-2-(2-oxobutyl)-6-(2-hydroxybutyl)-$\Delta^3$-piperideine] with tentative $^{13}C$ NMR spectrum assignments.

## RESULTS

Total alkaloid content (0.17 ± 0.01% dry weight, three replicates) was determined by titration of plant extracts on the basis of the molecular weight of compound 1 (Dollahite and Allen, 1962). Three major components were isolated from the *L. berlandieri* total extract. Compounds 1 and 2 were significantly more polar than compound 3. From MS, which is especially useful in structure determination of these compounds, the molecular weights were 241, 227, and 239, respectively (Table I), indicating possible molecular formulas of $C_{14}H_{27}NO_2$, $C_{13}H_{25}NO_2$, and $C_{14}H_{25}NO_2$. All three spectra showed prominent peaks at $m/z$ 94 and 96, indicating a common stable fragment with a possible formula of $C_6H_9N \pm H$. The $^1H$ NMR spectrum (Figure 1) of compound 1, which was available in the largest quantity, showed two methyl triplets at $\delta$ 0.89 and 0.90, indicating the presence of two terminal ethyl groups. A 3 H singlet at $\delta$ 2.27 indicated a methylamine derivative. Broad $^1H$ multiplets at $\delta$ 5.53 and 5.73 indicated a doubly substituted double bond. The $\delta$ 5.73 vinyl proton was shown by selective decoupling to be adjacent to two protons, giving a broad multiplet at $\delta$ 1.87 and 1.90, which in turn were coupled to an NCH at $\delta$ 3.25. The vinyl proton at $\delta$ 5.53 was coupled to the NCH at $\delta$ 3.10. Two multiplets centered at $\delta$ 3.54 and 3.64 were attributed to hydrogens on alcohol carbons. These were coupled neither to each other nor to the NCH hydrogens at $\delta$ 3.10 and 3.25 and were therefore at least two carbons distant from the rings. Since the chains terminate with ethyl groups, the side chains must both be 2-hydroxybutyl groups. The structure is shown in Figure 2. The $^{13}C$ NMR spectrum of 1 (Figure 2) shows vinyl carbons at $\delta$ 125.0 and 127.4 and amine carbons at $\delta$ 39.5, 49.1, and 62.7. Hydroxy carbons are found at $\delta$ 71.8 and 73.6. The mass spectral fragmentation pattern (Table I) showed prominent peaks at $m/z$ 168, 94, and 96, indicating loss of one or both side chains.

Compound 2 was spectrally similar to compound 1, the major difference being the replacement of the methyl group at $\delta$ 0.89 with a 3 H doublet at $\delta$ 1.14 (Figure 1). The mass spectrum shows major peaks at $m/z$ 168 and 154, fragments caused by the loss of side chains. Since the peak at $m/z$ 168 is of higher relative abundance, the shorter side chain was indicated as being in the allylic position. This was confirmed by $^1H$ decoupling studies at 500 MHz (Figure 3). The ring proton (NCH) at $\delta$ 3.33 was coupled to the ring methylene at $\delta$ 1.87–1.93 while the proton (NCH) at $\delta$ 3.22 was coupled both to the vinyl protons and to two methylene protons that were in turn coupled to the CHOH group ($\delta$ 3.88). The $\delta$ 3.88 peak exhibits coupling to the methyl doublet at $\delta$ 1.08. MS, therefore, can be used to show not only the length of the side chains but also their placement in relation to the double bond.

Compound 3, MW 239, gave an $^1H$ NMR spectrum similar to that of compound 1, but one of the two methyl triplets appeared as $\delta$ 1.14 (Figure 1). A 2 H quartet at $\delta$ 2.41 was coupled to this triplet, indicating the presence of an ethyl ketone. In the mass spectrum, the peak at $m/z$ 168 indicated loss of a four-carbon ketone-containing fragment. The $^1H$ NMR also showed an ABX pattern at $\delta$ 2.6, 2.8, and 3.14, which indicated one methylene separated the ketone from the piperideine ring. The $\delta$ 3.14

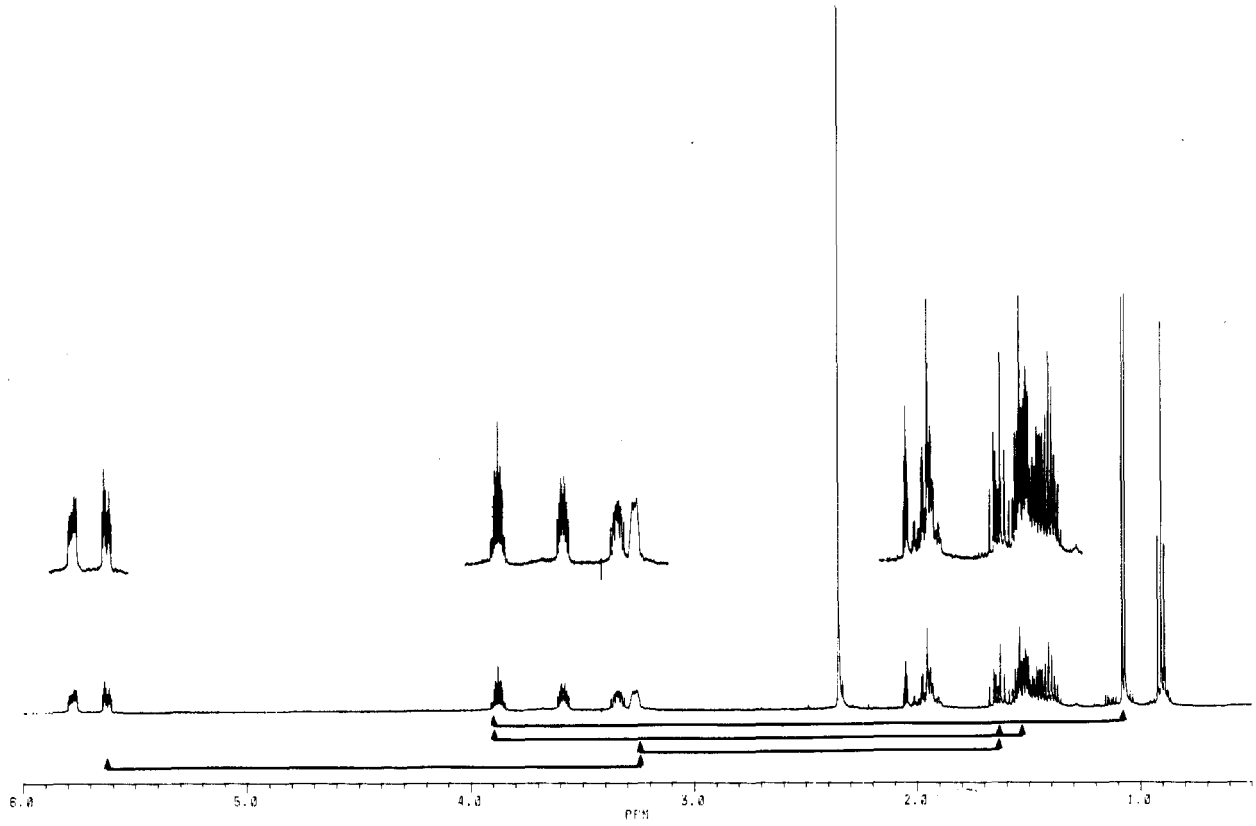
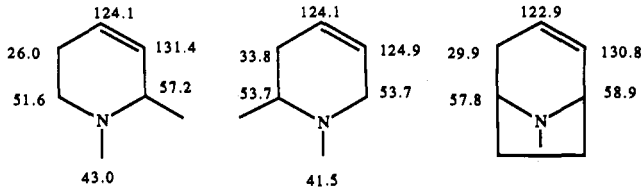



**Figure 3.** 500-MHz $^1$H NMR spectrum of compound **2** with important couplings noted. The methyl doublet at $\delta$ 1.08 is coupled to the C*H*OH at $\delta$ 3.88 which in turn is coupled to the $CH_2$ at $\delta$ 1.53 and 1.62. One of these protons is coupled to the NC*H* at $\delta$ 3.22, which is also coupled to the vinyl proton at $\delta$ 5.62. The three-carbon side chain is therefore adjacent to the double bond.

signal was also coupled to the $\delta$ 5.63 vinyl proton, indicating that the ketone side chain was in the allylic position. Its structure is therefore as shown in Figure 2. IR spectra provide little additional information but are consistent with the proposed structures in Figure 2. The carbonyl in compound **3** was confirmed by the presence of a band at 1710 cm$^{-1}$. All three compounds have similar spectra with bands consistent with aliphatic side chains [compound **1**, 2920, 2870, 2830 cm$^{-1}$; compound **2**, 2960, 2920, 2880 cm$^{-1}$; compound **3**, 2960, 2930, 2870 cm$^{-1}$], tertiary amines [compound **1**, 1260, 1180 cm$^{-1}$; compound **2**, 1230, 1190 cm$^{-1}$; compound **3**, 1225, 1200 cm$^{-1}$], and secondary alcohols [compound **1**, 3340 (br) cm$^{-1}$; compound **2**, 3360 (br) cm$^{-1}$; compound **3**, 3360 (br) cm$^{-1}$].

**Side Chain Stereochemistry.** In the carbon spectra (Figure 2), carbons attached to nitrogen in compounds **1** and **2** show similar shifts, much different from those of compound **3** and those of the model compounds.




(Wenkert et al., 1973) (Leete, 1977)

The proton spectrum shows a recognizable AB pattern for the ring methylene in compound **3**, but in compounds **1** and **2**, the methylene protons are more nearly shift equivalent. We therefore propose trans configuration for the side chains in compounds **1** and **2** and cis for those of compound **3**, pending comparison with a suitable trans model compound.

## DISCUSSION

No lobeline was found in the extracts in contrast to a previous report (Dollahite and Allen, 1962), but compound **1** was identified by Tschesche et al. (1961) in *Lobelia syphilitica* L. These alkaloids are related to lobinine (Wieland et al., 1931) and lobinanidine (Wieland et al., 1939).

Thin-layer and GC–MS chromatography of crude extracts indicated the presence of at least six compounds, the three described here plus three minor constituents. Compounds **1** and **3** account for approximately 90% of the alkaloidal material in the plant as estimated by thin-layer visualization and GC–MS chromatograms of crude plant extracts. Preliminary studies indicate that the crude extracts are toxic to laboratory animals (Kim, 1982), but the toxicities of individual alkaloids are unknown. The components(s) responsible for the toxicity to livestock also remains undetermined. Further toxicological studies are planned to clarify these points.

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# Protein Denaturation and Changes in Nucleotides of Fish Muscle during Frozen Storage

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Changes in quantity and composition of adenosine nucleotides and their relation to protein denaturation of fish muscle during frozen storage at −20 °C were studied. The protein denaturation was evaluated by the measurements of extractability of actomyosin (AM) and Ca-ATPase and Mg(EGTA)-ATPase activities of AM. The electrophoretic analysis of AM was also performed. No changes in electrophoretic separation of AM were observed during frozen storage. However, the molecular weight of myosin heavy chain (MHC) and actin decreased during frozen storage. The fish muscle with the highest levels of inosine (HxR) and hypoxanthine (Hx) and with the lowest levels of adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), and inosine 5′-monophosphate (IMP) was the most unstable in this study.

The deteriorative changes in texture as a consequence of long-term storage are considered to be due to protein denaturation during frozen storage (Dyer, 1951; Sikorski et al., 1976; Sikorski, 1978, 1980; Matsumoto, 1979, 1980; Shenouda, 1980; Andou et al., 1979, 1980; Noguchi, 1982; Acton et al., 1983; Jiang and Lee, 1985). For chub mackerel, pacific mackerel, and amberfish, much more denaturation of muscle proteins in fish frozen during postrigor than those frozen during prerigor was observed (Tsao et al., 1980; Fukuda et al., 1984). Many biochemical changes such as hydrolysis of lipids, oxidation of lipids, development of peptides and free amino acids, changes in nucleotide profile, etc., were involved in postmortis of fish muscle. The lipids and their derivatives, free amino acids, and peptides did affect the stability of muscle proteins during frozen storage (Noguchi, 1974; Sikorski et al., 1976; Sikorski, 1978, 1980; Matsumoto, 1979, 1980; Shenouda, 1980; Jiang, 1984; Jiang and Lee, 1985). However, the effect of adenosine nucleotides and interactions between proteins and nucleotides during frozen storage have not yet been studied. The present study aims to investigate the effect of adenosine nucleotides on protein denaturation so as to clarify the nature and mechanism of protein denaturation of frozen fish muscle.

As indicated in the *Data for Biochemical Research* (Burton, 1982; Symons, 1982), the p$K$ values for phosphate in adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), and inosine 5′-monophosphate (IMP) were 6.00–6.95, 6.10–6.70, 6.20–6.40, and 1.54–6.04, respectively. the p$K$ values for the adenosine base in these nucleotides were 4.00, 3.95, 3.74, and 8.90, respectively. However, the p$K$ values for the adenosine base in inosine (HxR) and hypoxanthine (Hx) were as follows: HxR, 1.20, 8.90; Hx, 1.98, 8.94, 12.10. According to the above data, ATP, ADP, AMP, and IMP should hold four, three, two, and two negatively charged groups at the pH condition of fish muscle (6.5–7.0), respectively. It is, accordingly, hypothesized that the repulsion force among protein molecules interacting with adenosine nucleotides with more than two negatively charged groups will be intensified and consequently will prevent protein denaturation during frozen storage, while that interacting with HxR and Hx will increase a little, but the possibility of hydrophilic interactions among protein molecules will increase due to the adenosine base.

## MATERIALS AND METHODS

Milkfish (*Chanos chanos*; body weight 310–380 g, length 34–38 cm) were obtained from a commercial culture farm in southern Taiwan. After being netted, the fish samples were iced and transported immediately to the laboratory. All fish were gutted, eviscerated, and headed. After the carcases were washed, fish samples were divided into three groups. Sample I was packed in polyethylene bags with five fish to each bag and stored at −20 °C. Samples II and III were stored at room temperature (25 °C) for 6 and 12 h and then in a −20 °C freezer for 18 weeks. at definite time intervals, one bag of each group was removed and thawed by immersion in running water (20–25 °C), until the body temperature reached 0 °C. The body temperature was measured by inserting a thermocouple into the center of dorsal portion. Five freeze–thawed fish from each

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