Natural Product Sciences
13(3) : 229-233 (2007)

Nucleoside Constituents of the Egyptian Tunicate *Eudistoma laysani*

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Abstract – Chemical investigation of the crude extract of the Egyptian marine tunicate *Eudistoma laysani* led to the isolation of a new nucleoside; 3-deazainosine and four known ones; inosine, 2'-deoxyuridine, adenosine and 2'-deoxyadenosine. Structural elucidation of the isolated compounds was based on intensive studies of their spectral data including 1D (^{1}H and ^{13}C) and 2D (^{1}H-^{1}H COSY, HMQC, HMBC) NMR together with mass spectra. The antioxidant effects of the isolated compounds were determined using DPPH, where they exhibited significant activities.
Keywords – Tunicates, *Eudistoma laysani*, nucleosides, spectroscopic techniques, antioxidant activity.

Introduction

Chordates are well represented in marine, fresh water and terrestrial habitats from the Equator to the high Northern and Southern latitudes. The phylum Chordata includes the well known vertebrates in addition to tunicates and lancelets. Many species of the marine tunicate *Eudistoma* were previously investigated and proved to be a rich source of a number of bioactive metabolites like indolocarbazole alkaloids (Schupp *et al.*, 1999 and 2002), β-carboline alkaloids (Adesanaya *et al.*, 1992; Van Wagoner *et al.*, 1999; Rashid *et al.*, 2001; Schupp *et al.*, 2003) and methylindole alkaloids (Makarieva *et al.*, 2001). In this work, the Egyptian marine tunicate *Eudistoma laysani* was subjected to successive fractionation using different chromatographic techniques to afford five nucleosides viz. inosine (**1**), 3-deazainosine (**2**), 2'-deoxyuridine (**3**), adenosine (**4**) and 2'-deoxyadenosine (**5**). This is the first report for the isolation of nucleosides from the genus *Eudistoma*.

Experimental

General experimental procedures – For column chromatography; silica gel (Merck, 70 - 230 mesh ASTM) and ODS (Merck Darmstadt) were used. Medium pressure pre-packed column Lobar Fertigsäule (250 × 10 mm) LiChroprep SiO_2 (40-63 μm) (Merck). Pre-coated silica gel 60 F-254 plates (Merck) were used for TLC.

Nuclear magnetic resonance analyses were recorded on Bruker Avance 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR; all the NMR data were recorded in DMSO-d_6. FAB mass spectral data were determined using API 2000 mass spectrometer.

Biological material – The tunicate *E. laysani* was collected by snorkeling at depth of 2 - 3 meters at Suez Canal, Egypt, in June 2005. It was identified by Prof. Francoise Monniot at MNHN. A voucher sample was deposited at MNHN, Paris, France under registration number: [A3-Eud-232] and in our Marine Invertebrate Collection at Suez Canal University under registration number: [BAL-4].

Extraction and Isolation – The fresh *E. laysani* sample (1.8 kg) was extracted with CH_2Cl_2-CH_3OH (1 : 1) till exhaustion. After concentration of the extract under vacuum, 6 g of the crude extract were successively fractionated on a flash column packed with ODS (20 cm L × 4 cm D) using H_2O and CH_3OH in gradient elution to obtain 3 main fractions, of which 2 fractions were investigated. The first fraction eluted with 100% H_2O (90 mg) was chromatographed on repeated silica gel column chromatography (32 cm L × 1.4 cm D) using CH_2Cl_2 and CH_3OH gradients to afford finally 10 mg of a white powder designated as compound **1** and 1.8 mg of a white creamy powder designated as compound **2**. The second fraction eluted with 10% CH_3OH (108 mg) from ODS column was chromatographed on a silica gel column (20 cm L × 1.4 cm D), eluted using CH_2Cl_2 and CH_3OH gradient. The portion (45 mg) eluted with CH_2Cl_2-CH_3OH

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(80 : 20) was purified using MPLC (25 cm L × 1 cm D, flow rate 0.15/5 min) which afforded two main sub-fractions: the first obtained by 10% CH_3OH in CH_2Cl_2, yielded compound **3** (3 mg, white powder); while the second sub-fraction eluted with 15% CH_3OH in CH_2Cl_2 was further purified on silica gel column chromatography (14 cm L × 1 cm D) eluted isocratically with CH_2Cl_2-CH_3OH (90 : 10) to obtain compound **4** (3.5 mg, white powder) and compound **5** (3.2 mg, white powder).

Negative FABMS obs. $[M-H]^+$ *m/z* 267.1, 266.1 and 227 for compounds **1**, **2** and **3**, respectively.

Positive FABMS obs. $[M+H]^+$ *m/z* 268.2 and 252.1 for compounds **4** and **5**, respectively ^{1}H-NMR and ^{13}C-NMR data were compiled: Tables **1** and **2**.

Determination of antioxidant activity of the isolated compounds – The five compounds were examined for their antioxidant activity using TLC autographic assay for DPPH radical scavenging effect (Takamatsu *et al.*, 2003). The compounds were dissolved in methanol at a concentration of 1 mg/mL and vitamin E was prepared at a similar concentration and used as a positive control. Six μg of each compound were applied in the form of a spot, 4 mm in diameter. The radical scavenging effects were detected on a TLC plate, using a spray reagent composed of 0.2 % (w/v) solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) in methanol. The plate was observed 30 min after spraying. All the compounds produced clear yellow spots against a purple background indicating a moderate antioxidant activity compared with vitamin E that gave an intense bright yellow zone. These findings coincide with previous investigation of the biological activity of inosine that ascertained its antioxidant effect (Gudkov *et al.*, 2006).

Results and Discussion

The structures of the isolated compounds were secured by 1D and 2D NMR studies and mass spectral determinations which indicated their nucleoside skeletons. Compound **1** was isolated as a white powder. Combined spectral data including 1D (^{1}H and ^{13}C) and 2D (^{1}H-^{1}H COSY, HMQC, HMBC) NMR as well as mass spectrum suggested the molecular formula of the compound to be $C_{10}H_{12}N_4O_5$ requiring seven degrees of unsaturation. ^{13}C-NMR spectrum showed resonances for ten carbons of which five were detected in the range from δ 61.2 - 87.3 indicating the oxygenated carbons of the sugar moiety. The sugar part was identified as ribose through comparison of the ^{13}C-NMR data with those reported for sugars (Agrawal, 1989) as well as from the 1D and 2D NMR data that confirmed its identity as ribose. The other five signals detected in the ^{13}C-NMR spectrum declared the presence of a carbonyl moiety (δ 156.4), two methines (δ 145.7 and 138.6) and two quaternary carbons (δ 148.1 and 124.3). The mass spectrum of compound **1** displayed a peak at *m/z* 134 $[M-H]^+$ for a hypoxanthine nucleotide. This postulation was ascertained from the ^{1}H- NMR spectrum that revealed two singlets at δ 8.09 and 8.34 for

Table 1. ^{1}H NMR data of compounds **1** - **5** [DMSO-d_6]

	δ H [mult., *J* (Hz)]				
Position	**1**	**2**	**3**	**4**	**5**
1-*NH*	12.4 (brs)	10.7 (brs)	11.30 (brs)	--	--
2	8.09 (s)	7.51 (brd, 8.1)	--	8.12 (s)	8.13 (s)
3	--	6.90 (brd, 8.1)	5.61 (d, 8)	--	--
4	--	--	7.86 (d, 8)	--	--
6-*NH*$_2$	--	--	--	7.31 (s)	7.30 (s)
8	8.34 (s)	8.10 (s)	--	8.33 (s)	8.33 (s)
1'	5.89 (d, 5.8)	6.1 (dd, 1.6, 6.3)	6.17 (t, 6.6)	5.88 (d, 7.2)	6.35 (dd, 7.7, 6.1)
2'	4.49 (q, 5.5)	4.75 (t, 5.4)	2.10 (m)	4.60 (q, 5.3)	2.72 (m) 2.27 (m)
2'-*OH*	5.50 (d, 5.9)	5.40 (brs)	--	5.46 (d, 7.0)	--
3'	4.13 (q, 4.8)	4.33 (m)	4.22 (brs)	4.13 (br d, 3.1)	4.41 (brs)
3'-*OH*	5.21 (d, 4.8)	5.27 (d, 4.0)	5.24 (d, 4.1)	5.20 (br d, 3.9)	5.31 (brs)
4'	3.94 (q, 3.8)	3.81 (m)	3.79 (d, 2.9)	3.94 (q, 3.1)	3.89 (m)
5'	3.58 (m) 3.64 (m)	3.52 (m)	3.58 (m)	3.58 (m) 3.67 (m)	3.52 (m) 3.62 (m)
5'-*OH*	5.09 (t, 5.6)	4.98 (t, 5.5)	5.00 (t, 5.1)	5.42 (t, 4.5)	5.25 (brs)

Table 2. ^{13}C NMR data of compounds **1 - 5** [DMSO - d_6]

	δ C (mult.)				
Position	**1**	**2**	**3**	**4**	**5**
2	145.7 (CH)	125.6 (CH)	150.3 (C)	152.3 (CH)	152.8 (CH)
3	--	102.4 (CH)	101.7 (CH)	--	–
4	148.1 (C)	135.5 (C)*	140.4 (CH)	149.0 (C)	149.3 (C)
5	124.3 (C)	133.1 (C)*	--	119.2 (C)	119.7 (C)
6	156.4 (C)	158.6 (C)	163.5 (C)	156.1 (C)	156.5 (C)
8	138.6 (CH)	138.9 (CH)	--	139.8 (CH)	139.9 (CH)
1'	87.3 (CH)	87.2 (CH)	84.1 (CH)	87.8 (CH)	84.4 (CH)
2'	74.0 (CH)	74.3 (CH)	40.1 (CH_2)	73.4 (CH)	40.4 (CH_2)
3'	70.2 (CH)	70.6 (CH)	70.3 (CH)	70.5 (CH)	71.4 (CH)
4'	85.5 (CH)	85.2 (CH)	87.3 (CH)	85.8 (CH)	88.4 (CH)
5'	61.2 (CH_2)	61.3 (CH_2)	61.2 (CH_2)	61.6 (CH_2)	62.3 (CH_2)

* Exchangeable values

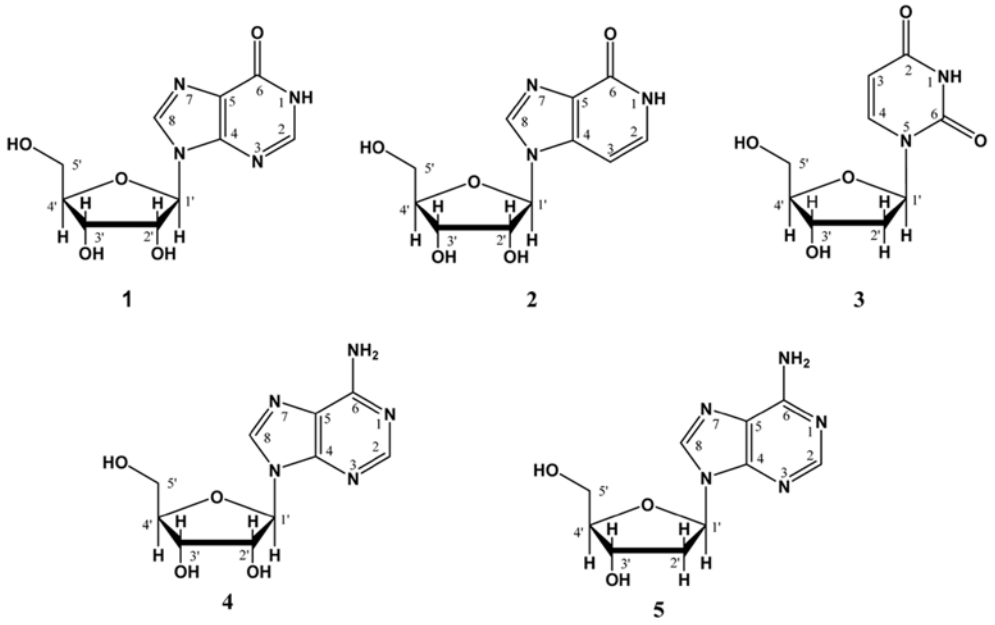

Fig. 1. Structures of compounds **1 - 5**.

two olefinic protons where the former was attributed to the proton of the imidazole ring. The previous discussion confirmed the chemical structure of compound **1** as inosine which was previously isolated from the sponge *Geodia baretti* (Lidgren and Bohlin 1988). Inosine was reported to be a potent stimulant of insulin release from the rat islets of Langerhans (Campbell and Taylor, 1977), possessing antioxidant and anti-inflammatory activities (Gudkov *et al.*, 2006; Buckley *et al.*, 2005) in addition to its neuroprotective effect (Hou *et al.*, 2004).

Compound **2** was a minor component isolated as a white creamy residue. The mass, ^{1}H- and ^{13}C-NMR spectral data of compound **2** suggested its molecular formula to be $C_{11}H_{13}N_3O_5$ requiring seven degrees of unsaturation. The ^{13}C-NMR spectrum showed resonances for eleven carbons, of which five were attributed to the sugar moiety that was found to be similar to that of compound **1** pointing to a ribose sugar. Comparing the NMR data of compound **2** with that of compound **1** indicated the replacement of a nitrogen atom in compound **1** by a methine group in compound **2** that was ascertained from the two broad doublets resonating at δ 6.90 and 7.51 coupled with J = 8.1 Hz, each integrated for one olefinic proton in compound **2** instead of the singlet detected at δ 8.09 in compound **1**. Interpretation of the ^{1}H- and ^{13}C-NMR spectral data confirmed the presence of

imidazole moiety through the characteristic singlet at δ 8.10 alongside with its corresponding carbon at δ 138.9 whereas the mass spectrum displayed a peak at *m/z* 133 $[M-H]^+$ for the imidazopyridinone moiety. From the previous discussion, the structure of compound **2** was deduced as 3-deazainosine. On reviewing the literature, this compound was previously mentioned as a synthetic one (Jiao *et al.,* 2002) and here is the first report for its isolation from a natural source.

Compound **3** was isolated as a white powder where both mass spectrum that displayed a pseudomolecular ion peak at *m/z* 227 $[M-H]^+$ and different NMR spectral data suggested the molecular formula to be $C_9H_{12}N_2O_5$, requiring five degrees of unsaturation. Both ^{1}H- and ^{13}C-NMR data proved the sugar part to be 2'-deoxyribose. The only difference noticed between the sugar part here and that of compound **1** was detected at C-2' through replacement of the hydroxyl group by a proton resonating at δ 2.10 as a multiplet with its corresponding carbon which was shifted up field to be detected at δ 40.1. ^{13}C-NMR spectrum displayed two signals at δ 163.5 and 150.3 for two carbonyl functionalities, while the two doublets detected at δ 7.86 and 5.61 coupled with J = 8.0 Hz in the ^{1}H-NMR spectrum were assignable for the two ortho olefinic protons of the nucleotide. These findings together with the 2D NMR data confirmed that compound **3** is 2'-deoxyuridine.

Compound **4** was isolated in the form of a white powder. The mass spectrum displayed a pseudomolecular ion peak at *m/z* 268 $[M+H]^+$ together with a characteristic base peak at *m/z* 136 $[M+H]^+$ assignable for the nucleotide adenine. This suggestion was confirmed from ^{1}H-NMR spectrum that revealed two singlets at δ 8.33 and 8.12 correlated with the carbon signals at δ 139.8 and 152.3, respectively. ^{13}C-NMR signals of the sugar part were found to be identical to those of compounds **1** and **2**, hence identified as ribose. From different 1D and 2D NMR data, compound **4** was unambiguously identified as adenosine which was previously isolated from the marine sponges *Clathria fasciculate* (Zhou *et al.*, 2005) and *Tethya aurantia* (Weber *et al.*, 1981) and also reported from the marine fungus *Aspergillus* sp. (Ouyang *et al.*, 2005). Adenosine was proved to have variable biological activities among them are the antiarrhythmic effect (Paul and Pfammater 1997), anti-inflammatory effect (Schrier *et al.,* 1990). In addition, it was reported as a strong inhibitor of insulin release from rat islets of Langerhans (Campbell and Taylor, 1982).

Compound **5** was isolated in the form of a white powder. Comparison of all spectral data of compound **5** with those of compound **4** indicated their structural similarity with few differences. The mass spectrum of compound **5** displayed a pseudomolecular ion peak at *m/z* 252 $[M+H]^+$ decreasing from that of compound **4** by 16 mass units for oxygen atom. The nucleotide moiety was identical to that of **4** and confirmed as adenine, while the sugar part was identified as 2'-deoxyribose. Hence, compound **5** was deduced as 2'-deoxyadenosine which was claimed to possess several activities among them is the paradoxical stimulation of human sperm motility, (Aitken *et al.*, 1986) and the inhibition of insulin release from rats islets of Langerhans (Campbell and Taylor, 1982).

Acknowledgement

The authors wish to acknowledge The Egyptian Academy of Scientific Research and Technology for funding this work under the project entitled "Bio-technological, Chemical and Genetic Approaches for Drug Discovery and Development from Red Sea Marine Organisms". Code No: MELF 05-1-016, G 132 HEL-113-33.

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(Accepted July 5, 2007)