ACETYLCHOLINESTERASE INHIBITING TRITERPENOIDAL ALKALOIDS FROM *BUXUS HYRCANA*

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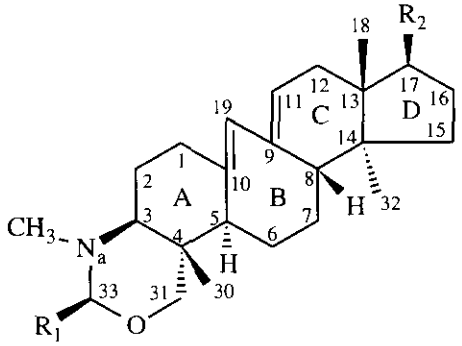
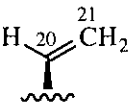
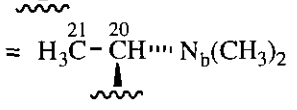
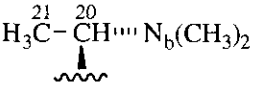
Abstract- *Buxus hyrcana* of Iranian origin has yielded two new triterpenoidal alkaloids (-)-hyrcanine (**1**) and (+)-homomoenjodaramine (**2**) along with a previously known compound, moenjodaramine (**3**), which has been obtained for the first time from this species. Compounds (**2**) and (**3**) were found to be potent acetylcholinesterase inhibitors.

Buxus species have long been known as rich sources of new and biologically active triterpenoidal alkaloids. Previous phytochemical studies have so far resulted in the isolation of more than 150 such compounds.[1,2] In the indigenous system of medicine, the extracts of genus *Buxus* are reported to be useful in various disorders such as malaria, rheumatism and skin infections.[3] Anti HIV activity was also reported from the ethanolic extract of *B. sempervirens* .[4] Continuing our investigations on the alkaloids of various *Buxus* species, we report here the isolation, structure elucidation and anti-acetylcholinesterase activity of two new compounds (-)-hyrcanine (**1**) and (+)-homomoenjodaramine (**2**) and of a known compound moenjodaramine (**3**), which has been isolated for the first time from this species. The structures of the new compounds were established with the aid of extensive spectroscopic studies including 2D-homo- and heteronuclear experiments (COSY-45°, HOHAHA, HMQC, HMBC).[5-8]



1 R_1=H, R_2= 

2 R_1=CH_3, R_2 = 

3 R_1=H, R_2 = 

(-)-Hyrcanine (**1**) was isolated as colourless fine needles. The EIMS displayed the molecular ion peak at *m/z* 381 which was further confirmed by FD and FAB +ve MS. The exact molecular weight was found to be *m/z* 381.3022 corresponding to the molecular formula $C_{26}H_{39}NO$, corresponding to eight degrees of unsaturation. The UV spectrum (λ_{max} 236 and 243 nm) was indicative of a 9(10→19) *abeo* diene system commonly found in *Buxus* alkaloids.[9,10] The IR spectrum afforded absorptions for NH (3401 cm^{-1}), CH (2903 cm^{-1}) and C=C (1642 cm^{-1}) functionalities.

The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) displayed three 3H-singlets at δ 0.68, 0.69 and 1.03 for the three tertiary methyl groups at C-18, C-32 and C-30 respectively. The vinylic H-11 and H-19 of the *abeo* diene system resonated at δ 5.58 (dd, J_1=2.5 Hz, J_2=2.4 Hz) and 5.99 (s) respectively. Two sets of AB doublets, one set appearing at δ 3.24 (d, $J_{31\alpha,31\beta}$=10.6 Hz) and 3.82 (d, $J_{31\beta,31\alpha}$=10.6 Hz) while the other set resonating at δ 3.60 (d, $J_{33\alpha,33\beta}$=7.6 Hz) and 4.45 (d, $J_{33\beta,33\alpha}$=7.6 Hz) were ascribed to the CH_2-31 and CH_2-33 α- and β-protons of the tetrahydrooxazine ring respectively. The N_a-CH_3 protons appeared as a 3H-singlet at δ 2.13. Besides these signals, there were three other downfield signals, a 1H-ddd at δ 5.76 ($J_{20,17}$=16.4, $J_{20,21b}$=10.6, $J_{20,21a}$=8.4 Hz) for CH-20 and two sets of double doublets at δ 4.94 (dd, $J_{21a,21b}$=2.1, $J_{21a,20}$=8.4 Hz) and 4.96 (dd, $J_{21b,21a}$=2.1, $J_{21b,20}$=10.6 Hz) due to H_a-21 and H_b-21 which were attached to a methine carbon (C-20, δ 140.5) and a methylene carbon (C-21, δ 114.4) (DEPT, HMQC). The ^{13}C-NMR spectrum ($CDCl_3$, 125 MHz, BB, DEPT) afforded resonances for 26 carbons (4 CH_3, 10 CH_2, 7 CH, 5C). The two quaternary carbons (C-10, C-9) of the *abeo*-diene system appeared at δ 132.3 and 138.3 whereas its two methine carbons (C-11, C-19) appeared at δ 130.6 and 131.2 respectively. The C-31 and C-33 carbons of the *N*-CH_2-O-CH_2-moiety resonated at δ 77.8 and 87.1 respectively. The HMQC spectrum served to establish direct one-bond ^{1}H-^{13}C connectivities of each protonated carbon in the molecule and the results are presented in Table 1.

The COSY-45° spectrum aided by the HOHAHA (20, 60 and 100 ms) spectra helped to unravel the different spin systems. These were then connected together with the help of long range ^{1}H/^{13}C correlations obtained from the HMBC spectrum. Important COSY-45° couplings were observed between H-31α/H-31β (δ 3.24/ 3.82), H-33α/H-33β (δ 3.60/ 4.45), H-3α/H-2 (δ 2.10/ 1.52, 1.85), H-2/H-1 (δ1.52, 1.85/2.25, 2.35) and between H-11/ H-12 (δ 5.58/ δ 1.80, 2.0). The HOHAHA spectra helped to identify the individual spin systems, starting from H-21 (δ 4.96, 4.94) which showed couplings with H-20 (δ 5.76), H-17 (δ 2.95), H-16 (δ 1.45, 1.55) and H-15 (δ 1.35). The N_a-CH_3 protons (δ 2.13), H-33 (δ 3.60, 4.45), H-31 (δ 3.24, 3.82) and H-30 (δ 1.03) showed long range heteronuclear interactions with C-3 (δ 71.2) in the HMBC spectrum. The N_a-CH_3 protons were also correlated with C-33 (δ 87.1) whereas H-31 and H-30 correlated with C-4 (δ 39.2), thereby revealing that the tetrahydrooxazine moiety is attached to C-3 and C-4. H-21 (δ 4.96, 4.94) showed long range interactions with C-20 (δ 140.5) and C-17 (δ 49.7) whereas H-20 (δ 5.76) correlated with C-17. These observations suggested that C-17 bears an olefinic group. This substitution pattern at C-17 has been observed for the first time in *Buxus* alkaloids.

The MS fragmentation pattern (HREIMS) was in agreement with the established structure. The loss of a methyl group from the M^+ resulted in the appearance of a peak at *m/z* 366.2783 ($C_{28}H_{36}NO$). Another

peak at *m/z* 127.0984 ($C_7H_{13}NO$) was due to the cleavage of ring A along with the *N*-methyl-tetrahydrooxazine ring-containing side chain.

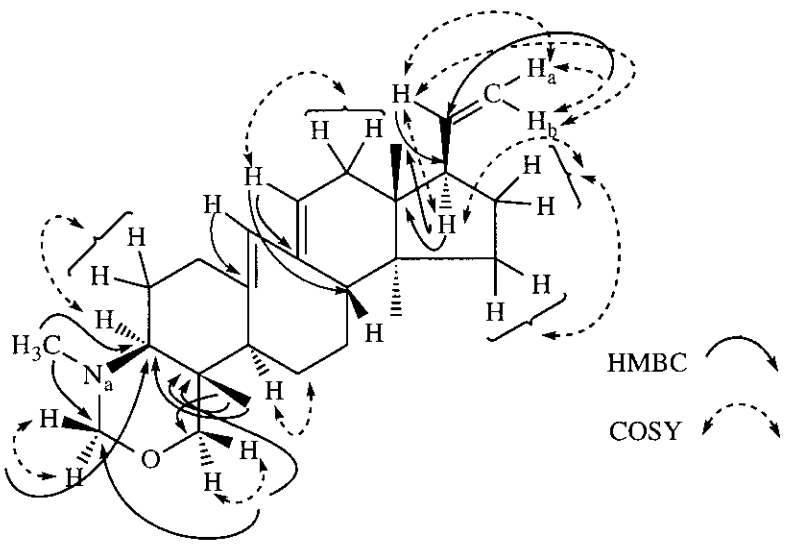


Figure 1: Important ^{1}H-^{1}H COSY and HMBC Correlations of **1**.

The α-stereochemistry of CH_2-31 substituted at C-4 was assigned on the basis of a previous observation that it is the α rather than the β methyl at C-4 which preferably undergoes oxidation.[11,12] H-3 and H-5 are invariably α-oriented while H-8 is always β-oriented in this class of alkaloids.[6] Based on these evidences structure **1** was assigned to this new alkaloid. The protonation of *N,N*-dimethylaminoethyl moiety of the *Buxus* alkaloids followed by the dehydroamination might result in the biosynthesis of **1**.

(+)-Homomoenjodaramine (**2**) was obtained as a white amorphous powder. The molecular formula was deduced to be $C_{29}H_{48}N_2O$, *m/z* 440.3750, by HREIMS while the molecular ion peak was observed at *m/z* 440 in the EI, FD and FAB +ve MS. The fragment ion at *m/z* 425.3500 ($C_{28}H_{45}N_2O$) was due to the loss of a methyl group from the M^+. The base peak at *m/z* 72.0806 ($C_4H_{10}N$) was characteristic of the nitrogen containing side chain cleavage from ring D.[13] The UV spectrum (λ_{max} 237 and 244 nm) was characteristic of a 9(10→19) *abeo* diene system. The IR spectrum was suggestive of the presence of an amino (3320 cm^{-1}) , CH (2802 cm^{-1}) and C=C (1595 cm^{-1}) functionalities. The ^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) revealed the presence of three tertiary methyl groups which appeared as 3H-singlets at δ 0.69, 0.74 and 1.00 for CH_3-18, CH_3-32 and CH_3-30 respectively. A 3H-doublet at δ 0.97 ($J_{21,20}$=6.4 Hz) was due to the secondary methyl group (CH_3-21). A 6H-singlet at δ 2.34 and a 3H-singlet at δ 2.12 were attributed to the N_b-dimethyl protons at C-20 and the N_a-CH_3 protons at C-3 respectively. Characteristic peaks for H-11 and H-19 appeared at δ 5.52 (m) and 5.98 (s) respectively. A set of AB doublets resonating at δ 3.28 (d, $J_{31\alpha,31\beta}$=10.7 Hz) and 3.76 (d, $J_{31\beta,31\alpha}$=10.7 Hz) was assigned to the CH_2-31 α- and β- protons respectively.

Table 1: ^{1}H and ^{13}C-NMR Chemical Shift Assignments of **1** and **2**.

Carbon	1		2	
	$\delta\ ^{13}C$(m*)	$\delta^{1}H$(*J*=Hz)	$\delta^{13}C$(m*)	$\delta^{1}H$(*J*=Hz)
1	38.9 (CH_2)	2.25 m, 2.35 m	27.0 (CH_2)	1.80 m, 1.92 m
2	26.7 (CH_2)	1.52 m, 1.85 m	25.3 (CH_2)	1.50 m
3	71.2 (CH)	2.10 m	70.6 (CH)	2.10 m
4	39.2 (C)		39.2 (C)	
5	48.4 (CH)	1.99 m	48.5 (CH)	1.90 m
6	25.1 (CH_2)	1.20 m	33.2 (CH_2)	1.45 m
7	25.5 (CH_2)	1.50 m	26.5 (CH_2)	1.90 m
8	50.0 (CH)	2.05 m	49.5 (CH)	2.10 m
9	138.3 (C)		138.3 (C)	
10	132.3 (C)		134.9 (C)	
11	130.6 (CH)	5.58 dd (2.4, 2.5)	128.5 (CH)	5.52 m
12	35.9 (CH_2)	1.80 m, 2.0 m	38.4 (CH_2)	1.99 m, 2.20 m
13	45.3 (C)		44.1(C)	
14	47.4 (C)		47.9 (C)	
15	28.9 (CH_2)	1.35 m	29.08 (CH_2)	1.22 m
16	33.8 (CH_2)	1.45 m, 1.55 m	39.5 (CH_2)	1.80 m, 1.90 m
17	49.7 (CH)	2.95 m	46.6 (CH)	1.80 m
18	16.7 (CH_3)	0.68 s	17.2 (CH_3)	0.69 s
19	131.2 (CH)	5.99 s	129.4 (CH)	5.98 s
20	140.5 (CH)	5.76 ddd (8.4, 10.6, 16.4)	64.5 (CH_3)	2.70 m
21	114.4 (CH_2)	4.94 dd (2.1, 8.4)	11.5 (CH_3)	0.97 d (6.4)
		4.96 dd (2.1, 10.6)		
30	13.8 (CH_3)	1.03 s	13.9 (CH_3)	1.00 s
31	77.8 (CH_2)	3.82 d (10.6), 3.24 d (10.6)	77.4 (CH_2)	3.76 d (10.7), 3.28 d (10.7)
32	17.1 (CH_3)	0.69 s	15.8 (CH_3)	0.74 s
33	87.1 (CH_2)	4.45 d (7.6),	92.0 (CH)	3.60 q (5.4)
		3.60 d (7.6)		
CH_3-N_a	36.2	2.13 s	38.0 (CH_3)	2.12 s
(CH_3)$_2$-N_b			40.0	2.34 s
N-CH$C\underline{H_3}$-O			20.7 (CH_3)	1.32 d (5.4)

m* multiplicity as determined by DEPT.

A quartet centered at δ 3.60 (q, $J_{33\alpha,C\underline{H_3}}$=5.4 Hz) was due to the H-33 vicinal to the methyl group which itself appeared as a doublet at δ 1.32 (d, $J_{C\underline{H_3},\ 33\alpha}$=5.4 Hz). The ^{13}C-NMR spectra ($CDCl_3$, 125 MHz,

BB, DEPT) displayed 29 peaks for seven methine, eight methylene, nine methyl and five quaternary carbons. Direct one bond $^{1}H/^{13}C$ connectivities of all the protonated carbons were determined from the HMQC experiment (Table 1).

In the COSY-45° spectrum strong cross-peaks were observed between H-3α (δ 2.10)/H-2 (δ 1.50), H-2/H-1 (δ 1.80, 1.92), H-5α (δ 1.90)/H-6 (δ 1.45), H-7 (δ 1.90)/H-8 (δ 2.10), H-11 (δ 5.52)/H-12 (δ 1.99, 2.20) and between H-21 (δ 0.97) and H-20 (δ 2.70). In the HMBC spectrum H-21 exhibited shift correlation with C-20 (δ 64.5). The N_b-dimethyl protons (δ 2.34) showed correlations with C-20 and C-16 (δ 39.5). Similarly H-11 (δ 5.52) was found to be correlated with C-8 (δ 49.5) and C-13 (δ 44.1), whereas H-19 (δ 5.98) exhibited long range correlations with C-10 (δ 134.9), C-5 (δ 48.5) and C-4 (δ 39.2). The N_a-CH_3 protons (δ 2.12) afforded interactions with C-33 (δ 92.0) which in turn was also correlated with the methyl protons (δ 1.32) as well as with H-3 (δ 2.10). H-30 (δ 1.00) displayed HMBC interactions with C-3 (δ 70.6), C-4 (δ 39.2) and C-31 (δ 77.4) indicating that positions 3 and 4 are the sites of attachment of the substituted tetrahydroxazine moiety.

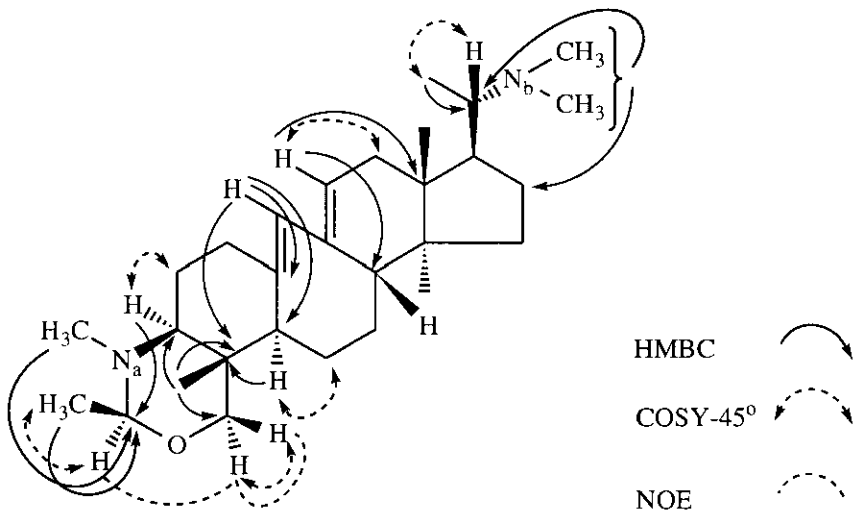


Figure 2: Important ^{1}H-^{1}H COSY, NOE and HMBC correlations of **2**.

The β-orientation of the methyl group substituted at C-33 was deduced from the NOE difference studies. Irradiation at δ 3.28 (H-31α) caused an enhancement (45.8%) of the signal at δ 3.76 (H-31β) and enhancement (18.3 %)of another signal at δ 3.60 (H-33), which established the α-stereochemistry of H-33. Hence the CH_3 group should be β-oriented.

Along with these two new compounds a previously known compound (+)-moenjodaramine (**3**) was also obtained from the leaves of *B. hyrcana*. The spectral data (^{1}H-NMR, MS, UV, IR) of **3** matched well with the reported literature values.[14]

Some plants of the family *Buxaceae* have exhibited antiacetylcholinesterase properties.[15] Furthermore

buxaminol-E, a steroidal alkaloid isolated from *B. sempervirens*, has also been reported as a good inhibitor of the enzyme.[16] We therefore tested our *Buxus* alkaloids for antiacetylcholinesterase activity. Inhibition of acetylcholinesterase (AChE) is considered as a promising approach for the treatment of Alzheimer's disease and for possible therapeutic applications in the treatment of Parkinson's disease, ageing and myasthenia gravis.[17,18] The concentration of compounds **2** and **3** that inhibited the enzyme activity by 50% (IC_{50}) are presented in Table 2 while the effect of concentration on % inhibition is depicted by a bar graph (Figure 3). Eserine ((-)-physostigmine, IC_{50} = 61 nM) was used as a positive control.

Table 2: *In vitro* quantitative inhibition of acetylcholinesterase by **1**-**3**.

Compound	IC_{50} (μM)*
(-)-hyrcanine (**1**)	Inactive
(+)-homomoenjodaramine (**2**)	19.2±0.32
(+)-moenjodaramine (**3**)	50.8±0.812

* IC_{50} are the mean ± standard mean error of five assays.

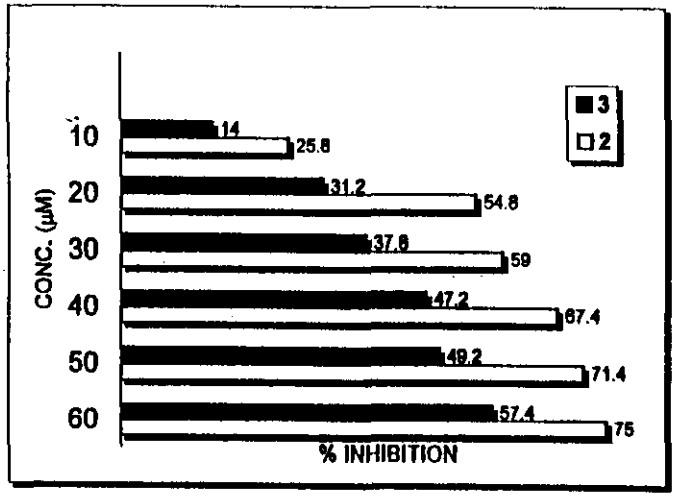


Figure 3: Effect of varying conc. of **2** and **3** on % inhibition of acetylcholinesterase.

EXPERIMENTAL

General Experimental Procedures. The melting point was determined on a Buchi 510 melting point apparatus and is uncorrected. Optical rotations were measured on JASCO DIP-360 digital polarimeter. UV and IR spectra were recorded in MeOH on Shimadzu UV-240 and in $CHCl_3$ on Shimadzu IR-240 spectrophotometers respectively. ^{1}H-NMR spectra were recorded in $CDCl_3$ at 400 MHz and 500 MHz on Bruker AM-400 and AMX-500 NMR spectrometer. ^{13}C-NMR was recorded at 125 MHz on Bruker AMX-500 NMR spectrometer. HREIMS were recorded on a JEOL-JMS HX 110 mass spectrometer. Column chromatography was carried out using silica gel (E. Merck, type 60, 70-230 mesh) and prep. TLC was conducted on pre-coated silica gel GF-254 preparative plates (20 x 20 cm, 0.25 mm thick).

Extraction and Isolation. The leaves (28 kg) of *B. hyrcana* were collected from Tehran, Iran during March-April 1997. Extraction was carried out with MeOH (30 L x 3) at 25°C for 3 days. The solvent was evaporated in *vacuo* to afford a gum (3 kg) which was then suspended in H_2O. This aqueous layer after defatting with hexane (10 L x 3), was extracted with $CHCl_3$ (10 L x 3) at different pH values. The fraction obtained at pH 9.0 (188.0 g) was subjected to column chromatography (silica gel, 70-230 mesh, 1880 g). The column was eluted with mixtures of $CHCl_3$-MeOH in order of increasing polarity. Elution with $CHCl_3$ afforded impure **1** which was further purified by prep. TLC using hexane: $CHCl_3$:Et_2NH (90:10:1). Elution with $CHCl_3$-MeOH (95:05) afforded a fraction (12.55 g) which was again subjected to column chromatography (silica gel, 70-230 mesh, 360 g) employing hexane: ether: Et_2NH as eluting solvent. This afforded **2** as an amorphous powder (30 mg).

(-)-Hyrcanine (1). Colourless needles (acetone); m p 118°C, $[\alpha]_D^{25}$: -33° (c=0.095, $CHCl_3$), UV (MeOH) λ_{max} (log ε): 243 (4.01), 236 (4.00) nm, IR ($CHCl_3$) ν_{max}: 3401 (NH), 2903 (CH), 1642 (C=C) cm^{-1}, ^{1}H-NMR ($CDCl_3$, 500 MHz): Table **1**, ^{13}C-NMR ($CDCl_3$, 125 MHz): Table **1**; HREIMS *m/z* (rel. int. %): 381.3022 ($C_{26}H_{39}NO$, 100), 366.2783 ($C_{25}H_{36}NO$, 27), 127.0984 ($C_7H_{13}NO$, 30).

(+)-Homomoenjodaramine (2). White amorphous powder; $[\alpha]_D^{25}$: +40° (c=1.12, $CHCl_3$); UV(MeOH) λ_{max} (log ε):244 (3.85), 237 (3.82) nm; IR ($CHCl_3$) ν_{max}: 3320 (NH) 2802 (CH), 1595 (C=C) cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz): Table **1**; ^{13}C-NMR ($CDCl_3$, 125 MHz): Table **1**; HREIMS *m/z* (rel. int. %): 440.3750 ($C_{29}H_{48}N_2O$, 69), 425.3500 ($C_{28}H_{45}N_2O$), 72.0806 ($C_4H_{10}N$, 100).

Acetylcholinestrase Inhibition Assay. Electric eel acetylcholinesterase, acetylthiocholine, 5,5'-thiobis-2-nitrobenzoic acid (DTNB) and eserine ((-)-physostigmine) were purchased from Sigma (St. Louis, MO). Buffers and other chemicals were of extra pure analytical grade. Acetylcholinesterase inhibition was determined spectrophotometrically using acetylthiocholine as substrate by modifying the method of Ellman.[19] The reaction was carried in 100 mM. Sodium phosphate buffer was used (pH 8.0) at 25 °C. In a typical assay 165 µL buffer, 10 µL enzyme preparation and 5 µL test compound solution were mixed and incubated for 30 min. 10 µLof DTNB (5,5'-dithiobis-2-nitrobenzoic acid) was added and the reaction was then started by adding 10 µL of acetylthiocholine. The hydrolysis of acetylthiocholine was determined by monitoring the formation of the yellow 5-thio-2-nitrobenzoate anion (as a result of the reaction of 5,5'-dithiobis-2-nitrobenzoic acid with thiocholine released by the enzymatic hydrolysis of acetylthiocholine) at a wavelength of 412 nm.

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