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Structural characterization of 13²-hydroxy-(13²-*S*)-phaeophytin-a from leaves and stems of *Amaranthus tricolor* isolated by high-speed countercurrent chromatography

Gerold Jerz ^{a*}, Tabiwang N. Arrey ^a, Victor Wray ^b, Qizhen Du ^c, Peter Winterhalter ^a

^a Institute of Food Chemistry, Technical University of Braunschweig, Schleinitzstrasse 20, 38106 Braunschweig, Germany.

^b Department of Structural Biology, Helmholtz Centre for Infection Research (HZI), Inhoffenstraße 7, D-38124 Braunschweig, Germany.

^c Research Center of Bioactive Products for Food, Zhejiang Gongshang University, Hangzhou, 310035, China.

* corresponding author: g.jerz@tu-bs.de, phone: +49-531-391-7208, fax: +49-531-391-7230

Abstract

Amaranthus tricolor (Amaranthaceae) is a popular vegetable in China. Separation of lipophilic extracts of the aerial parts of *A. tricolor* by means of preparative ‘High-speed Countercurrent Chromatography’ (HSCCC) led to the isolation of two chlorophyll breakdown products which were characterized as 13²-hydroxy-(13²-*S*)-phaeophytin-a (**1**) and chlorophyll b methoxylactone (**2**).

The structure of **1** was established using a combination of 1D- and 2D-NMR spectroscopic and mass spectrometric techniques. Component **2** was identified by means of ESI-MS/MS analysis.

Keywords:

Amaranthus tricolor, Amaranthaceae, 13²-hydroxy-(13²-*S*)-phaeophytin-a, chlorophyll b methoxylactone, Countercurrent chromatography, 1D- and 2D-NMR spectroscopy, EI-MS, ESI-MS/MS.

Industrial relevance: Chlorophylls and their degradation products are natural pigments of interesting bioactivities. For industry, recovery of these pigments from waste materials of fruit and vegetable processings could be a promising by-product line. Here we present the isolation of a pure chlorophyll breakdown product which is in principle suited as a nutraceutical ingredient. Large-scale isolation procedures using countercurrent chromatography techniques for chlorophyll related pigments are currently under development.

1. Introduction

Amaranthus tricolor (Amaranthaceae) is a popular vegetable in China where the leafy plant material is cooked in a similar way to spinach. The plant is well known for its content of purple betalain pigments, such as amaranthine and the isomer isoamaranthine. In this study, however we have focussed on extractable lipophilic bioactive natural products.

The consumption of vegetables is an important factor in the human diet. During storage and processing of such foods, chlorophylls are converted into phaeophytins, phaeophorbides and pyropheophytins. Beside the chlorophylls, degradation products are also known to be beneficial to health in prevention of age-related diseases. Different biological activities of the porphyrins have been documented: e.g. strong antioxidant activities for phaeophytins are known for the lipophilic extracts of green tea *Camellia sinensis* (Higashi-Okai, Taniguchi, & Okai, 2000). They have also been reported to act as cytostatic substances in ‘*in-vitro*’ models and could be potent agents in treatment of malignant tumors, and in cancer prevention (Nakatani, Ourisson, & Beck, 1981; Chernomorsky, Segelman, & Poretz, 1999). The antimutagenic capacity of chlorophyll and its derivatives against strong mutagens has been documented in the ‘Ames’-*Salmonella* system (Chernomorsky et al., 1999; Okai & Higashi-Okai, 1997/98).

Using an all-liquid *high-speed countercurrent chromatography* technique (HSCCC) we were able to fractionate and purify 13²-hydroxy-(13²-*S*)-phaeophytin (**1**) – an allomerization product of chlorophyll a. Structure elucidation was performed using 1D-NMR (¹H, ¹³C, DEPT135), 2D-NMR (HMBC, HMQC, ROESY) spectroscopy, combined with molecular weight determinations by EI-MS (70 eV), ESI-MS/MS fragmentation experiments and high resolution ESI-MS. ESI-MS/MS analysis of a second pure HSCCC fraction indicated the presence of the rare chlorophyll b methoxylactone (**2**) (Fig. 1).

2. Materials and Methods

2.1. Plant Material and Extraction

Lyophilized leaves and stems of *Amaranthus tricolor* (1500 g) were harvested close to Hangzhou (China, 2004). Exhaustive maceration with fresh *n*-hexane resulted in extracts which were dried and stored under a nitrogen atmosphere at -25°C.

2.2. High-speed countercurrent chromatography (HSCCC)

The separation of the *n*-hexane extract of *Amaranthus tricolor* (stems and leaves) was carried out on a ‘*high-speed countercurrent chromatograph*’ (HSCCC) model CCC-1000 (Pharma-Tech Research Corp., U.S.A.) equipped with three preparative coils made of PTFE tubing (2.6 mm i.d. x 160 m),

which were connected in series (850 mL total volume). The separation was run at a revolution speed of 850 rpm applying a two-phase solvent system composed of *n*-hexane - MeOH (2:1, v/v). All solvents were of analytical grade and were delivered by a Biotronik HPLC pump BT 3020 (Jasco, Gross-Umstadt, Germany).

An optimized solvent system was selected after preliminary partitioning experiments of low amounts of crude extract (Ito & Conway, 1996). Rapid separation of the phases occurred indicating the potential stability of the highly lipophilic solvent system under high-speed countercurrent conditions. Preparation of the biphasic solvent system was achieved by thorough equilibration of the solvent components in a separatory funnel at ambient temperature. The resulting two phases were separated shortly before use and degassed by ultrasonication. Elution mode of the system was 'head to tail', using the upper organic phase as stationary phase and the lower aqueous phase as mobile phase. The multilayer coiled column was initially completely filled with the upper organic phase. After rotation at 850 rpm, the sample solution (900 mg) was dissolved in 26 mL of a mixture of upper and lower phase and introduced into the separation teflon column through an injection loop (25 mL). The lower phase was pumped into the head end of the HSCCC coil column at a flow-rate of 3 mL min⁻¹. Peak monitoring of the effluent stream from the tail outlet of the coil-column was performed with a UV-detector (Knauer, K-2501) at λ 254 nm using a preparative cell and collecting fractions into test tubes with a fraction collector (Pharmacia, Superfrac, Sweden) at 4-min intervals. After separation, the solvent in the coil column was ejected with nitrogen gas and 40 % of the stationary phase was retained. Analysis of CCC fractions was performed by TLC and UV-light detection. Purity of recovered fractions **a** - **c** was investigated by electrospray ionization (ESI) MS analysis.

2.3. Mass-spectrometry analysis (ESI-MS, HR-ESI-MS and EI-MS)

ESI-LC-MS, and MSⁿ experiments were performed on a BRUKER Esquire LC-MS/MS ion trap system in positive and negative ion mode introducing the samples via a syringe pump: flow rate 350 μ L h⁻¹; mass range: 50-2200 amu, drying gas N₂ (7.0 L min⁻¹, 330 °C), nebulizer pressure (5 psi), Cap: 4500V, End plate: 4000V, Cap exit: 90 V, Cap exit offset 60V, Skim 1 30V, Skim 2 10V, Trap drive 60. MS-MS experiments afforded fragmentation amplitude values between 0.8 and 1.2.

HR-ESI-MS of **1** was measured on a MAT Trap hybrid tandem mass spectrometer (Thermo-Finnigan, Bremen, Germany). EI-MS (70eV) measurement of **1** was performed by direct inlet on a triple quadrupole mass spectrometer TSQ-7000 (MAT-Finnigan).

2.4. Nuclear magnetic resonance (NMR) analysis

^1H , ^{13}C , and DEPT-135 NMR spectra were recorded in MeOH-d_4 at 25 °C on a Bruker AMX 300 NMR spectrometer (Karlsruhe, Germany; ^1H 300 MHz; ^{13}C 75.5 MHz). 2D-NMR experiments (HMQC, HMBC, $^1\text{H}/^1\text{H}$ -COSY, ROESY) were performed on a Bruker AVANCE DMX 600 NMR spectrometer. Chemical shifts (δ) are reported in ppm relative to the residual solvent signals (δ_{H} 7.26 and δ_{C} 77.36 ppm) and coupling constants (J) in Hz..

3. Experimental data

13²-hydroxy-(13²-*S*)-phaeophytin-a (**1**): $\text{C}_{55}\text{H}_{74}\text{N}_4\text{O}_6$: EI-MS (70eV) m/z (rel. int.): 886.5 [M]⁺ (70), 827.4 [M-COOCH_3]⁺ (20), 607.2 [M-phytol]⁺ (39), 549.1 [M-phytol-COOCH_3]⁺ (21), 476.1 (12), 256.2 (21), 123 (52), 95.1 (100);

ESI-MS (pos) m/z 909.8 [M+Na]⁺, ESI-MS/MS (909.8): m/z 631, 613, 587, 581, 569, 555, 537, 511; ESI-MS (neg) m/z 885.7 [M-H]⁻, 683.5, 652.2, ESI-MS/MS (885.7): 854, 826, 606 [M-H-phytol]⁻, 547, 535, 515, 503, 487, 460; HR-ESI-MS (pos) m/z (rel. int.): 909.55029 [M+Na]⁺, calc. $\text{C}_{55}\text{H}_{74}\text{N}_4\text{O}_6\text{Na}$ 909.55054; ^1H -NMR (300 MHz, CDCl_3): porphyrine moiety cf. Table 1, Phytol-moiety: δ [ppm]: 0.83-0.88 (12H, *m*, CH_3 -16, 17, 18, 19), 1.63 (3H, *d*, CH_3 -20), 1.93 (2H, *t*, $J=7.5$ Hz, H-4), 4.59 (2H, *d*, $J=7.5$ Hz, H-1), 5.24 (1H, *t*, $J=7.0$ Hz, H-2). ^{13}C -NMR (75.5 MHz, CDCl_3): porphyrine moiety cf. Table 1, Phytol-moiety: δ [ppm]: 16.7 (CH_3 , C-20), 20.0 (CH_3 , C-19), 20.1 (CH_3 , C-18), 23.0 (CH_3 , C-17), 23.1 (CH_3 , C-16), 24.8 (CH_2 , C-9), 25.1 (CH_2 , C-13), 25.4 (CH_2 , C-5), 28.3 (CH , C-15), 33.0 (CH , C-7), 33.1 (CH , C-11), 37.0 (CH_2 , C-6), 37.6 (CH_2 , C-8), 37.7 (CH_2 , C-12), 37.8 (CH_2 , C-10), 39.7 (CH_2 , C-14), 40.2 (CH_2 , C-4), 61.9 (CH_2 , C-1), 118.4 (CH_2 , C-2), 143.0 (C, C-3).

chlorophyll b methoxylactone (**2**): ESI-MS (pos) m/z 975.8 [M+Na]⁺, ESI-MS/MS (975.8): m/z 960 [M+Na-CH_3]⁺, 814, 681 [$\text{M+Na-CH}_3\text{-Phytol}$]⁺, 519.

4. Results and discussion

4.1. HSCCC separation

The aim of the present study was to investigate the lipophilic extracts of *Amaranthus tricolor* by means of high-speed countercurrent chromatography (HSCCC) for the recovery of chlorophyll related pigments. HSCCC as an all-liquid chromatographic system that functions without solid support, and separation is based on fast partitioning effects of the analytes between two immiscible liquid phases (Ito & Conway, 1996). Irreversible adsorbing effects and artefact formation is minimized.

For the preparative isolation by HSCCC different biphasic solvent systems of high lipophilicity were evaluated. The best results for equal partitioning of *Amaranthus tricolor* constituents between the two

immiscible phases were obtained with a system composed of *n*-hexane - MeOH (2:1 v/v). The separation was performed in the 'head-to-tail' mode using the lower aqueous solvent layer as mobile phase. The selected solvent system exhibited retention of 40 % of organic stationary phase in the HSCCC coil-system during the separation. Lipophilic natural products of strong emulsifying capacity, i.e. phospholipids and free fatty acids hamper the separation of the two phases in the HSCCC unit and are the reason for the steady loss of stationary phase during the chromatographic run.

In our study, 900 mg of the *n*-hexane crude extract was dissolved in the two-phase solvent mixture and injected to the preparative HSCCC system (Ito & Conway, 1996). The resulting *peak a* consisted of a complex mixture of components, such as triglycerides and free fatty acids.

Peak b had to be purified from the phytosterol α -spinasterol using a short silica gel column to yield 5 mg of the allomeric substance 13²-hydroxy-(13²-*S*)-phaeophytin (**1**). Noteably for the HSCCC-separation is the achieved baseline separation between peak b and peak c (chlorophyll b methoxylactone, **2**) (cf. Fig. 2).

4.2 Determination of chemical structures

EI-MS (70eV) analysis of hydroxy-phaeophytin-a (**1**) resulted in a molecular ion signal $[M]^+$ at m/z 886. The observed isotope ratio suggested the central Mg ion was absent (van Breemen, Canjuna, & Schwartz, 1991). ESI-MS (neg) resulted in $[M-H]^-$ m/z 885, and ESI-MS (pos) $[M+Na]^+$ yielded m/z 910, and suggested a hydroxy-phaeophytin for substance **1**. In the case of the ESI-MS (pos), a strong MS/MS fragment ion at m/z 631 $[M+Na-C_{20}H_{38}]^+$ indicated the cleavage of a phytadiene moiety ($\Delta m/z$ 279) (Gauthier-Jaques, Bortlik, Hau, & Fay, 2001; Eckardt, Keeley, & Maxwell 1991). Another fragment ion at m/z 613 showed the cleavage of water due to hydroxylation at C-13². In the HR-ESI-MS a strong signal abundance for **1** was also achieved in the positive ionization mode resulting in a sodium addition to the quasimolecular ion peak $[M+Na]^+$ at m/z 909.55029 (calc. C₅₅H₇₄N₄O₆Na 909.55054).

The ¹H-NMR showed all resonances of the phaeophytin-a backbone: three aromatic methyl groups (δ 3.30, 3.45, 3.77 ppm), a methoxyl group (δ 3.62 ppm) and a vinyl substitution (δ 6.23, 6.32, 8.03 ppm) with a characteristic exomethylene coupling pattern (cf. Tab. 1). The attached phytol moiety was recognized by a large number of overlapping proton signals of aliphatic methylene and methyl functions. Indicative proton resonances for the phytol moiety were the carbinol resonances CH₂-1 (δ 4.59 ppm), the olefinic proton H-2 (δ 5.24 ppm) and the methyl group CH₃-16 (δ 1.63 ppm) (Fig. 1).

Of stereochemical relevance was the ¹H singlet signal of 13²-OH at δ 5.47 ppm suggesting a 13*S* configuration stereochemistry in good agreement with reference data (δ 5.53 ppm: Matsuo, Ono, Hamasaki, & Nozaki 1996; Nakatani, Ourisson, & Beck, 1981). The co-existence of the diastereomer

13*R* hydroxy-phaeophytin was excluded as no signal was observed at δ 5.35 ppm (Matsuo et al., 1996; Nakatani et al., 1981) (cf. Tab. 1).

Nuclear Overhauser enhancements in the ROESY spectrum of **1** indicated a through-space connectivity due to a close spacial proximity between the protons of the methyl-ester group OCH₃-13⁴ (δ 3.62 ppm) and H-17 (δ 4.17 ppm) (cf. Fig. 3, 4; Tab. 1) corroborating the 13*S*-configuration in the hydroxy-phaeophytin-a structure. Due to the inflexibility of the porphyrin system, the observed nuclear Overhauser enhancements are only possible for a 13*S*-configuration. Significant ROESY signals were also observed for the aromatic methyl groups (CH₃-2¹, 7¹, 12¹), olefinic (H-5, 10, 20), vinyl (CH-3¹, CH₂-3²) and ethyl protons (CH₂-8¹, CH₃-8²) verifying the assignments of all ¹H-NMR signals in the porphyrine system (Fig. 3, 4; Tab. 1).

Thirty five carbon signals were detected in the ¹³C NMR spectrum for the porphyrine system of **1**, while the aliphatic phytol moiety afforded a partly overlapping set of signals in the aliphatic region. Overall ¹H- and ¹³C-NMR data for the phytol moiety of **1** were in accordance with the literature data (Nabeta, Kawae, Kikuchi, Saitoh, Okuyama (1995); Itoh, Kawano, & Nabeta, 2003).

Direct ¹³C-NMR detection of imine carbons of the porphyrin system was hampered by low signal intensities. However the shifts of some of these could be confirmed from the ¹H-inversely detected HMBC experiment that showed long-range HC-correlations to some imine carbons (cf. Fig. 5). The nature of all carbons (CH, CH₂, CH₃, quarternary C) (cf. Table 1) was determined by a standard DEPT-135 experiment.

Hydroxylation of ring E was deduced from the carbinol resonance C-13² at δ 89.4 ppm (Matsuo et al., 1996) and the quarternary nature was confirmed from the DEPT-135 experiment.

The HMQC experiment allowed all ¹*J*CH connectivities to be deduced (cf. Tab. 1). All observed two- and three-bond CH-connectivities (HMBC) were conclusive and confirmed the proposed structure of the 13²-hydroxy-(13²-*S*)-phaeophytin-a (**1**) and are presented in Figure 5 and Table 1.

For complete analysis of the porphyrine structure in **1** all detected ^{2,3}*J*CH long-range correlations of the olefinic protons H-5, H-10, H-20 afforded the connectivities between the four pyrrole rings (Fig. 5). The signal of H-5 (δ 9.60 ppm) showed correlations to C-3 and C-4 (ring A) and also to C-7 of ring B. Similarly, H-10 (δ 9.76 ppm) showed connectivities to C-8 (ring B) and to C-12 of ring C, and H-20 (δ 8.76 ppm) to C-18 (ring D) and C-1 and C-2 (ring A).

The constitution of ring D was deduced from long-range signals from H-17 (δ 4.17 ppm) to C-19, C-18 and C-18¹ and from H-18 (δ 4.56 ppm) to C-16, C-17¹, C-20. Interestingly, protons of CH₃-12¹ (δ 3.77 ppm) showed a ⁴*J*-CH correlation to the carbonyl function C-13¹ (δ 192.2 ppm) in ring system E. The ³*J*-CH correlation of OCH₃-13⁴ (δ 3.62 ppm) to the carboxyl group C-13³ (δ 173.3 ppm) confirmed the position of the methyl-ester.

Hydroxylation of ring E in the phaeophytin-a system **1** was confirmed by two important ³*J*-correlations observed for the proton of the OH-group at C-13² (δ 5.47 ppm) to C-13³ and C-15.

The attachment of the phytol-moiety to the porphyrine system was indicated by the 3J CH correlation of the carbinol CH₂-1 (δ 4.59 ppm) to the carboxyl function C-17³, and similar correlations confirmed the position of the double bond in the phytol moiety.

Peak *c* of the HSCCC separation (Fig. 2) resulted in a second chlorophyll related pigment (10 mg). Interestingly, the ESI-MS (pos) showed a very high molecular weight of *m/z* 975 due to quasimolecular ion [M+Na]⁺ formation. ESI-MS/MS (pos) gave abundant fragment ions [M+Na-15]⁺ at *m/z* 960, and *m/z* 681 [M+Na-CH₃-C₂₀H₃₈]⁺. From these data we suggest peak *c* is the chlorophyll b methoxylactone (**2**) with the molecular weight of Mr 952 (Verzegnassi, Riffé-Chalard, & Gülaçar, 2000). Further NMR studies are in progress for structural confirmation.

5. Conclusions

To date the high speed countercurrent chromatography technique has been mainly used for isolation of polar natural pigments such as anthocyanins. Here we demonstrated that HSCCC is also a versatile tool for isolation of fluorescent and non-fluorescent chlorophyll breakdown products from crude lipophilic plant extracts such as those from *Amaranthus tricolor* (Amaranthaceae). To the best of our knowledge 13²-hydroxy-(13²-*S*)-phaeophytin-a (**1**) and chlorophyll b methoxylactone (**2**) were recovered for the first time from plant material of *Amaranthus tricolor*. Isolation of pure chlorophyll related pigments as reference material will facilitate future nutritional and medicinal studies for this class of bioactive components.

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Figure 1. Structures of 13²-hydroxy-(13²-*S*)-phaeophytin-a (**1**), and chlorophyll b methoxylactone (**2**).

Figure 2. HSCCC chromatogram of the *n*-hexane extract of *Amaranthus tricolor* leaves and stems. The HSCCC was operated in ‘head-to-tail’ mode. Two-phase solvent system: *n*-hexane - methanol 2 : 1 (v/v). *Peak a*: complex mixture of various compounds, *Peak b*: 13²-hydroxy-(13²-*S*)-phaeophytin a (**1**), *Peak c*: chlorophyll b methoxylactone (**2**).

Figure 3. Structure relevant ROESY correlation signals observed in **1** showing proton-proton through-space interactions in the porphyrin-ring system, and elucidating the (13²-*S*)-configuration.

Figure 4. Relevant Nuclear Overhauser signals (δ 0.00 – 5.50 ppm) in the ROESY-experiment of 13²-hydroxy-(13²-*S*)-phaeophytin-a (**1**) for elucidation of the (13²-*S*)-configuration.

Figure 5. Structure relevant long-range HC-correlation signals observed in the HMBC of **1**.

Table 1. ^1H -, ^{13}C -, DEPT-NMR, ROESY and $^{2,3}J\text{-CH}$ long-range correlations (HMBC) for compound **1** (δ [ppm]; J [Hz]) in CDCl_3 (phytyl moiety cf. 3. Experimental data).

Position	^1H	ROESY	^{13}C	DEPT	$^{2,3}J\text{-CH}$
1			142.8	C	
2			132.6	C	
2 ¹	3.45 <i>s</i>	H-3 ¹ , H-3 ² (<i>E</i>), H-20	12.4	CH ₃	C-1, C-2, C-3
3			137.0	C	
3 ¹	8.03 <i>dd</i> (11.5, 17.5)	$\text{CH}_3\text{-}3^2$ (<i>Z</i>), $\text{CH}_3\text{-}2^1$	129.4	CH	C-3 ² , C-3, C-2
3 ² (<i>E</i>)	6.32 <i>dd</i> (17.5, 1.0)	$\text{CH}_3\text{-}2^1$, H-5	123.6	CH ₂	C-3 ¹ , C-3
3 ² (<i>Z</i>)	6.23 <i>dd</i> (11.5, 1.0)	H-3 ¹			C-3 ¹
4			136.3	C	
5	9.60 <i>s</i>	$\text{CH}_3\text{-}7^1$, H-3 ² (<i>E</i>)	98.4	CH	C-3, C-4, C-7
6			150.9	C	
7			136.3	C	
7 ¹	3.30 <i>s</i>	$\text{CH}_3\text{-}8^1$, $\text{CH}_3\text{-}8^2$, H-5	11.6	CH ₃	C-6, C-7, C-8
8			145.3	C	
8 ¹	3.77 <i>q</i>	$\text{CH}_3\text{-}7^1$	19.9	CH ₂	C-8 ² , C-8
8 ²	1.70 <i>t</i>	$\text{CH}_3\text{-}7^1$	17.6	CH ₃	C-8 ¹ , C-8
9			155.4	C	
10	9.76 <i>s</i>	$\text{CH}_3\text{-}12^1$	104.7	CH	C-12, C-8
11			138.4	C	
12			129.4	C	
12 ¹	3.77 <i>s</i>	H-10	12.7	CH ₃	C-12, C-11, C-13, C-13 ¹
13			127.7	C	
13 ¹			192.2	C	
13 ²			89.4	C	
13 ² -OH	5.47 <i>s</i>				C-15, C-13 ³
13 ³			173.3	C	
13 ⁴ -OCH ₃	3.62 <i>s</i>	H-17	53.8	CH ₃	C-13 ³
14			150.9	C	
15			107.1	C	

16			164.0	C	
17	4.17 <i>m</i>	^{13}C -OCH ₃ , CH ₂ -17 ¹ , CH ₂ -17 ² , CH ₃ -18 ¹	52.6	CH	C-19, C-18 ¹ , C-17 ²
17 ¹	H _A 2.25 <i>m</i> H _B 2.95 <i>m</i>	CH ₂ -17 ² , H-17	31.6	CH ₂	C-17 ³ C-17, C-17 ² , C-18
17 ²	2.55	CH ₂ -17 ¹ , H-17	32.0	CH ₂	C-17 ³ , C-17 ¹ , C-17
17 ³			173.8	C	
18	4.56 <i>m</i>	H-20, CH ₃ -18 ¹	50.9	CH	C-16, C-17, C-20, C-17 ¹ , C-18 ¹
18 ¹	1.63 <i>d</i> (7.0)	H-17, CH ₂ -17 ¹ , H-20	22.9	CH ₃	
19			173.0	C	
20	8.76 <i>s</i>	CH ₃ -2 ¹ , H-18, CH ₃ -18 ¹	95.0	CH	C-1, C-2, C-18