Preparation of ^{13}C-24-methylcholesta-5,24(28)-dien-3β-ol by cultivation of the Baikal diatom *Synedra acus* in $NaH^{13}CO_3$

A. I. Kalinovsky,[a] A. G. Gorshkov,[b] L. P. Ponomarenko,[a] V. A. Stonik,[a] P. S. Dmitrenok,[a] and M. A. Grachev[b]*

[a] *Pacific Institute of Bioorganic Chemistry, Far-Eastern Branch of the Russian Academy of Sciences, 159 prosp. 100-letiya Vladivostoka, 690022 Vladivostok, Russian Federation. Fax: +7 (423) 231 4050. E-mail: stonik@piboc.dvo.ru*

[b] *Limnological Institute, Siberian Branch of the Russian Academy of Sciences, 3 ul. Ulan-Batorskaya, 664033 Irkutsk, Russian Federation. Fax: +7 (395) 242 5405. E-mail: gorchkov_ag@mail.ru*

A method for preparing ^{13}C-24-methylcholesta-5,24(28)-dien-3β-ol (24-methylenecholesterol) with ^{13}C enrichment degree of up to 55% was proposed. The method is based on cultivation of the freshwater diatom alga *Synedra acus* in a medium containing sodium ^{13}C-bicarbonate as the carbon source. ^{13}C-24-Methylenecholesterol isolated for the first time was studied by ^{13}C NMR spectroscopy and mass spectrometry.

Key words: diatom microalga *Synedra acus*, sodium ^{13}C-bicarbonate, steroids, ^{13}C-24-methylcholesta-5,24(28)-dien-3β-ol, ^{13}C NMR spectroscopy, mass spectrometry.

Biomolecules labeled by stable isotopes find extensive use in biomedical studies. Chemical and enzymatic methods for their preparation are complicated and expensive. An alternative approach is cultivation of microorganisms in the media containing labeled precursors followed by isolation of the target biosynthesis products. The cost of labeled compounds obtained by the biotechnological technique is usually lower than the cost of the products of organic synthesis.[1]

Diatom algae are of considerable practical interest for the biotechnology of organic compounds with a unique structure. This is due to the fact that the total structure of their genome has been decoded (the marine species *Thalassiosira pseudonana* and *Pheodactylum tricprnutum*) and more than 11000 genes were identified,[2] and also various methods for diatom cultivation in photobioreactors under controlled conditions were developed.[3,4] The diatom algae produce a variety of steroids, which can be represented by 3—10 compounds in isolated sterol fractions.[5] However, ^{13}C-cholesterol is the only sterol present in the market of ^{13}C-labeled compounds. In this work we propose a method for preparing ^{13}C-24-methylenecholesterol based on cultivation of the freshwater diatom alga *Synedra acus* in a medium with sodium ^{13}C-bicarbonate.

Experimental

^{13}C NMR spectra were recorded on a Bruker DRX-500 spectrometer in $CDCl_3$ with internal $SiMe_4$. Column chromatography was carried out on silica gel (50—160 μm, Sorbpolymer, Russia). TLC was carried out on Sorbfil plates (Sorbpolymer, Russia), the spots were visualized by a H_2SO_4—EtOH mixture (1 : 4, v/v) followed by heating. GLC/MS analysis was performed on a HP6890-HP5973 GC-MS System chromatograph (Hewlett Packard) with an HP-5MS capillary column at 270 °C using He as the carrier gas and an ionizing voltage of 70 eV. EI mass spectrum of a ^{13}C-24-methylcholesta-5,24(28)-dien-3β-ol acetate sample with maximum ^{13}C inclusion was recorded on an AMD-604S double focusing magnetic-sector mass spectrometer (AMD-Intectra, Germany), electron energy 70 eV, accelerating voltage 8 kV. The ^{13}C-isotope enrichment factor (*IEF*) was determined by mass spectrometry and calculated from the following equation:[6]

$$IEF(\%) = \frac{100}{n}\left[\frac{M \cdot I_M + (M+1) \cdot I_{M+1} + \ldots + (M+x) \cdot I_{M+x}}{I_M + I_{M+1} + \ldots + I_{M+x}} - M\right],$$

where M, $M + 1$ and so on are the numerical m/z values for molecular peaks of different isotopomers; I_M, I_{M+1} and so on are the integral intensities of the signals corresponding to the ions of these masses, n is the number of C atoms in the molecule, x is the number of ^{13}C atoms in the molecule.

The following commercial chemicals were used: labeled bicarbonate $NaH^{13}CO_3$ (Cambridge Isotope Laboratories, Inc.) with ^{13}C isotope purity of at least 99%; $Ca(NO_3)_2 \cdot 2H_2O$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $NaH^{12}CO_3$, H_3BO_3, $MnCl_2 \cdot 4H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, $Na_2SiO_3 \cdot 9H_2O$, $FeCl_3$ (all analytically pure grade, Reakhim); Na_2EDTA (biotech. grade, Hilicon); cyanocobalamin (Vitamin B_{12}), thiamine hydrochloride (Vitamin B_1) (both analytically pure grade, OJSC Veropharm); biotin (Applichem). Water used for preparing the medium was boiled for 2 h to remove CO_2.

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The laboratory culture of diatom *Synedra acus subsp. radians* obtained from the Baikal phytoplankton was the starting material for cultivation.[7]

The biomass *S. acus* was cultivated in 1-Liter sterile flasks tightly plugged by a sterile cotton pellet at ~20 °C with stirring at intervals under natural alternation of day and night in the DM medium (see Ref. 8) of the following composition, mg L^{-1}: $Ca(NO_3)_2 \cdot 4H_2O$, 20; KH_2PO_4, 12.4; $MgSO_4 \cdot 7H_2O$, 25; $NaH^{13}CO_3$, 16; Na_2EDTA, 2.25; H_3BO_3, 2.48; $MnCl_2 \cdot 4H_2O$, 1.39; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.0; cyanocobalamin, 0.04; thiamine hydrochloride, 0.04; biotin, 0.04; $Na_2SiO_3 \cdot 9H_2O$, 42.6; $FeCl_3$, 1.6. The microalgae *S. acus* were inoculated by adding an aqueous suspension of the cells (~5 mL) into a flask with a culture medium (950 mL) to obtain a culture concentration of ~12000 colonies mL^{-1}. After 5—6 days of cultuvation, 50 mg of sodium ^{13}C-bicarbonate was added. The pH value was maintained at 7.7—8.0 by adding required amounts of concentrated HCl. After 10 days of cultivation, the content of the flasks was filtered, and 0.9—1.0 g of crude biomass *S. acus* was obtained from each flask. To obtain biomass with higher enrichment, ^{13}C-enriched inocula were used; after their inoculation, the cell concentration did not exceed 3000 colonies mL^{-1}.

The sterol fraction was isolated by successive extraction of crude biomass (~5 g) with EtOH (15 mL), EtOH—EtOAc mixture (1 : 1, v/v) (15 mL), and EtOAc (2×15 mL). The extracts were combined and concentrated *in vacuo*, the concentrates were chromatographed on a column with silica gel in the *n*-C_6H_{14}—EtOAc system (10 : 1, v/v). Sterols were identified in the obtained fractions by TLC. Sterol-containing fractions were combined and concentrated to dryness on a rotary evaporator. The residue was acetylated with acetic anhydride in pyridine (1 : 1) for 24 h at ~20 °C, and the reagents were removed *in vacuo*. Sterol acetates were purified by column chromatography on silica gel in the *n*-C_6H_{14}—EtOAc system (100 : 1) to give 5.6 mg of the sterol acetate fraction.

Results and Discussion

The composition of the sterol fractions in the *S. acus* obtained by cultuvating this alga in a medium with $NaH^{13}CO_3$ is summarized in Table 1. Samples *1* and *2* were cultivated using inocula grown in a medium with $NaH^{12}CO_3$ and $NaH^{13}CO_3$, respectively. The sterol fraction of sample *2* was found to contain only ^{13}C-24-methylenecholesterol distinguished by a high isotope enrichment factor (*IEF* = 55%). The detected sterols were identified by GLC/MS.

Table 1. Content (*c*) of the sterol fraction in *S. acus* and ^{13}C isotope enrichment factors (*IEF*) of sterols

Sterol	*c* (%) [*IEF* (%)]	
	Sample *1* [a]	Sample *2* [b]
Cholesterol	5.3 [1.0][c]	—
24-Methylenecholesterol	91.1 [36]	100 [55]
24-Ethylcholesta-5,22-dien-3β-ol	3.5 [0.9][c]	—
24-Ethylcholest-5-en-3β-ol	Traces [0.9][c]	—

[a] The inoculum was grown in a $NaH^{12}CO_3$ medium.
[b] The inoculum was grown in a $NaH^{13}CO_3$ medium.
[c] The isotope enrichment factor of about 1% corresponds to the natural abundance of ^{13}C isotope.

^{13}C-24-Methylenecholesterol with high ^{13}C inclusion in the structure was obtained for the first time; therefore, study of its spectral characteristics, in particular, ^{13}C NMR spectra was important not only to confirm its structure and to determine the isotope distribution but also to measure the $^1J_{C,C}$ constants. For non-labeled or selectively labeled compound, these constant cannot be measured due to low probability that two or more ^{13}C atoms will be found in the neighboring positions of a molecule.

The proton-decoupled ^{13}C NMR spectrum (Fig. 1, *a*, *b*) of 24-methylenecholesterol acetate (*IEF* = 55%) confirms the uniform inclusion of ^{13}C in each position of sterol molecule (from C(1) to C(28)). Instead of the singlets for C atoms usually observed in the spectra of this type, the ^{13}C NMR spectrum exhibits multiplets. These multiplets consist of a central line due to the ^{13}C atom in the corresponding position of the molecule[9] having no other ^{13}C atoms in the neighborhood, which is superimposed by the central line of the ^{13}C—^{13}C—^{13}C triplet or quintet and a series of satellite lines arising from the spin-spin coupling of this ^{13}C atom with ^{13}C atoms in each neighboring position. Comparison of the intensities for signals of these two types allows one to estimate the total inclusion of the label as being substantially higher than 50%.

For detailed analysis, we chose the signals of six C atoms with relatively simple multiplicity (each comprises three lines), namely, the signals $C(18)H_3$, $C(19)H_3$, $C(21)H_3$, $C(26)H_3$, $C(27)H_3$, and $C(28)H_2$ (δ_C: 11.9, 19.3, 18.7, 21.85, 22.0, 105.9, respectively). The ratio of the areas of central and satellite lines in the spectrum, which is always 1 : 2, means that the number of $\mathbf{H_3{}^{13}C-{}^{13}C}$ (or $\mathbf{H_2{}^{13}C={}^{13}C-}$) groups is up to twice as great as the number of $\mathbf{H_3{}^{13}C-{}^{12}C}$ (or $\mathbf{H_2{}^{13}C={}^{12}C-}$) groups. All signals of methylene C atoms comprise five lines, those of methine atoms comprise seven lines, and the signals of quaternary C atoms include nine lines.

The ^{13}C NMR spectra were useful for determining the $^1J_{C,C}$ constants, which have not been determined previously for 24-methylenecholesterol or other sterols (see Table 2).

The EI mass spectrum of ^{13}C-24-methylenecholesterol acetate (Fig. 2) is distinguished by high signal multiplicity, which characterizes the qualitative and quantitative composition of the compound isotopomers. In the series of $[M - AcOH]^+$ peaks with *m/z* from 380 to 407 (Fig. 3), the isotopomer with *m/z* 407 attests to the maximum inclusion of ^{13}C isotopes in the sterol structure: 27 of the possible 28, and the highest intensity of the signals of

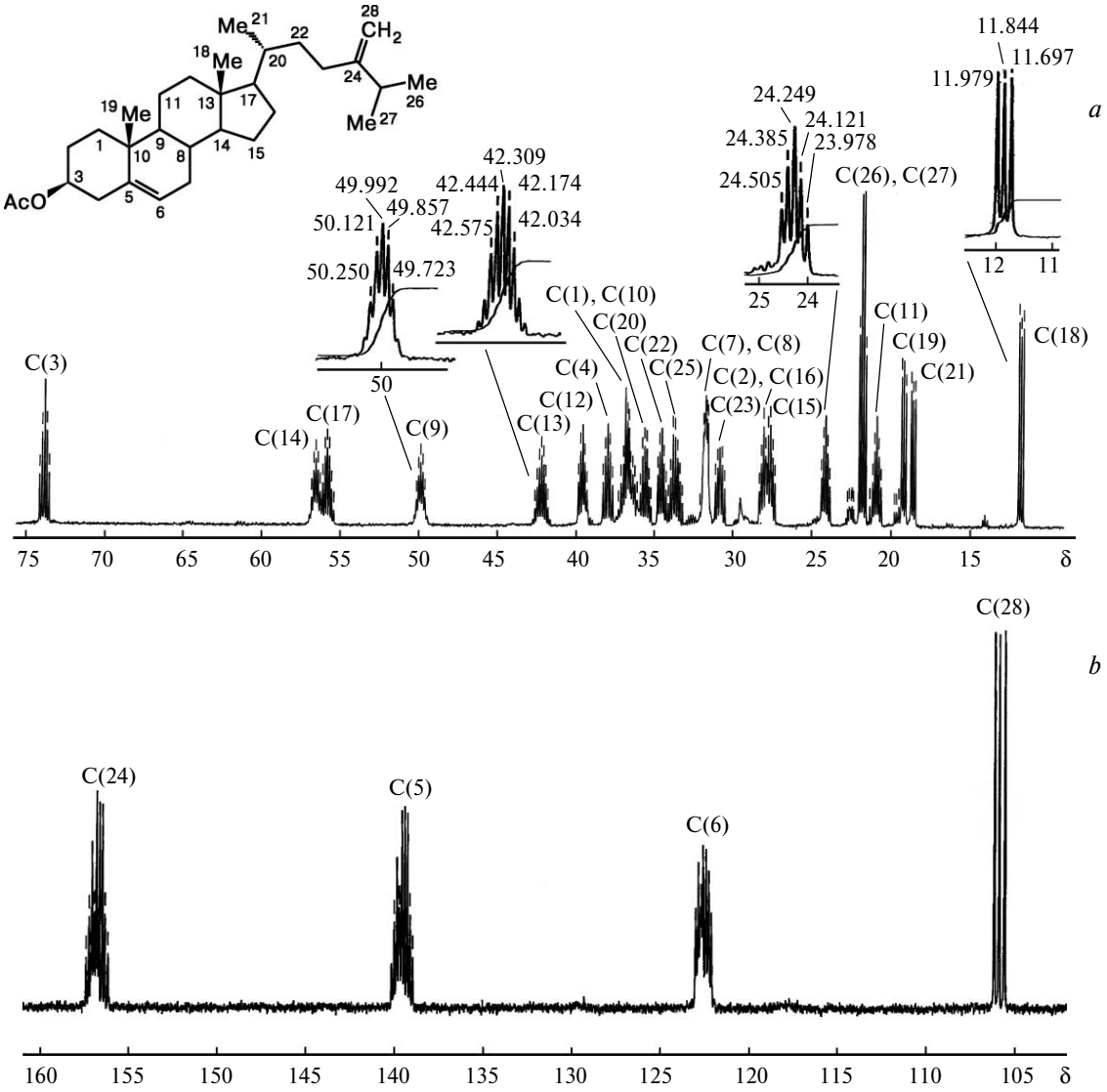

Fig. 1. Fragments of the ^{13}C NMR spectrum of 24-methylenecholesterol acetate, *IEF* = 55% (sample *2*): δ 10—75 (*a*), 100—160 (*b*).

the isotopomers with *m/z* 399—401 points to predominance of molecules with 19—21 ^{13}C atoms in the structure. Therefore, the integral *IEF* of sterol does not exceed 55%. The peak with *m/z* 296 in the mass spectrum of sterol caused by the McLafferty type rearrangement with abstraction of the C(23)—C(28) fragment and elimination of acetic acid appears as a series of peaks with *m/z* from 300 to 317 in which the peak with *m/z* 317 is indicative of ^{12}C to ^{13}C replacement of 21 of the 22 carbon atoms.

All other characteristic signals in the mass spectrum are also responsible for the corresponding series of isotope peaks. By analyzing their qualitative and quantitative composition, it is possible to estimate the number of isotopes in fragment ions. Note that using a similar approach for analysis of mass spectra, it is possible to check assumptions concerning the fragmentation paths of natural compounds if a fully ^{13}C-labeled sample of the compound is available and its EI mass spectrum has been recorded.

Table 2. Some $^1J_{C,C}$ spin-spin coupling constants in the spectrum of ^{13}C-labeled 24-methylenecholesterol acetate with high ^{13}C contents

$^1J_{C,C}$	Value/Hz	$^1J_{C,C}$	Value/Hz	$^1J_{C,C}$	Value/Hz
$J_{15,14}$	31.1±1.8	$J_{12,11}$	34.0±1.6	$J_{18,13}$	35.1±1.2
$J_{15,16}$	31.1±1.8	$J_{12,13}$	34.0±1.6	$J_{3,2}$	37.1±0.8
$J_{9,8}$	33.0±0.9	$J_{19,10}$	34.0±1.6	$J_{3,4}$	37.1±0.8
$J_{9,10}$	33.0±0.9	$J_{21,20}$	34.0±1.6	$J_{4,5}$	38.6±1.3
$J_{9,11}$	33.0±0.9	$J_{17,13}$	35.1±1.2	$J_{6,5}$	73.4±1.8
$J_{14,13}$	33.0±0.9	$J_{17,20}$	35.1±1.2	$J_{28,24}$	73.4±1.8
$J_{14,8}$	33.0±0.9	$J_{17,16}$	35.1±1.2		

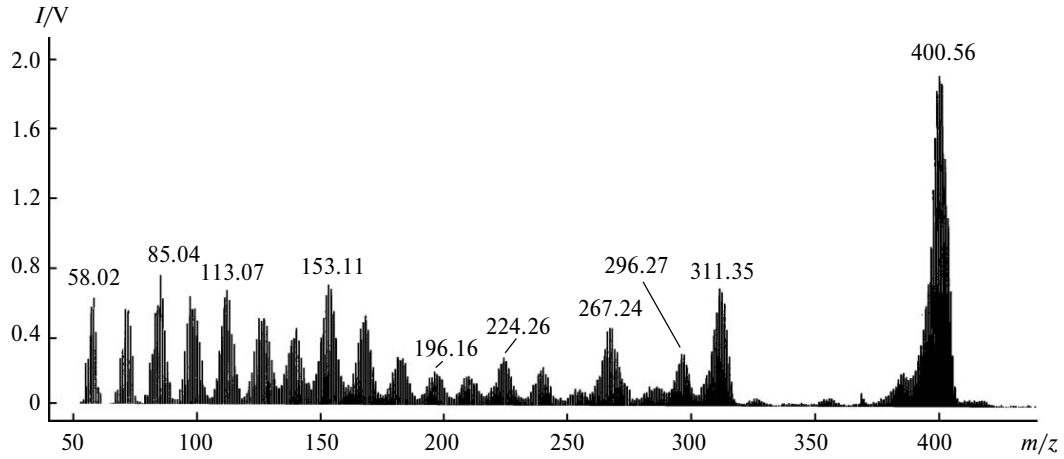

Fig. 2. EI mass spectrum of 24-methylenecholesterol acetate, *IEF* = 55% (sample *2*).

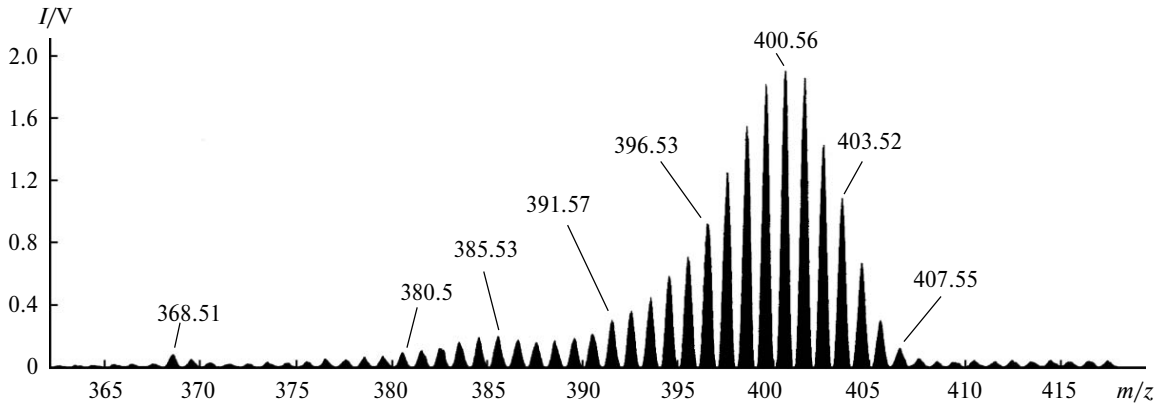

Fig. 3. Fragments of the EI mass spectrum of 24-methylenecholesterol acetate corresponding to the $[M - AcOH]^+$ ion peaks.

24-Methylenecholesterol labeled with a stable isotope is a convenient biochemical tool for investigating the metabolic transformations of C_{28}- to C_{27}-sterols *via* dealkylation, which occurs in insects and other invertebrate organisms. In addition, there are numerous highly active polar steroids with 24(28)-methylene side chain, in particular, brassinolide dolichosterones and dolicholides from higher plants, some asterosaponins and polyhydroxysteroids from starfishes,[10] and steroid disulfates of brittle stars.[11] Mechanisms of their biosyntheses can be established by using labeled sterol as the biosynthetic precursors.

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