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Usage of Relative Differences of Experimental and Calculated Vicinal Constants $^3J_{\mathrm{HH}}$ for Conformational Analysis of Rigid Molecules in Liquid

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Abstract Comparison of the relative differences between the experimental and calculated values of proton–proton vicinal scalar constants obtained from nuclear magnetic resonance spectra of two structurally similar organic molecules makes it possible to increase the efficiency of using Karplus relationship for experimental detection and quantitative description of the conformational distortions in molecules under investigation. Advantages and disadvantages of the proposed approach are considered on the examples of the study of two pairs of rigid steroid molecules in solution where they are in a single conformation. These steroids have potential biological activity which is determined by their structural and conformational features. There is only one structural difference in each pair of compared steroids. The influence of small modifications such as substituent variation on the molecules spatial structure was investigated by the joint use of molecular optimization methods (semiempirical and molecular mechanic) to determine the dihedral angles and Karplus-type equation of C.A.G. Haasnoot, F.A.A.M. de Leeuw and C.A. Altona to calculate vicinal constants in ethane fragments. It was shown that the usage of relative differences of experimental and calculated vicinal constants for conformational analysis of rigid molecules eliminates systematic errors of vicinal constant calculations. Such approach allows us to detect small distortions between conformations of comparable molecules with high accuracy, which are concluded in the differences of the corresponding dihedral angles within not more than 10°–15°. The proposed approach is a more sensitive way of studying small specific features of the spatial structure

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of molecules in comparison with the known methods on the basis of Karplus-type equation in which the absolute values of experimental and calculated vicinal proton–proton constants are compared.

1 Introduction

Successful application of nuclear magnetic resonance (NMR) spectroscopy in structural and conformational analysis of molecules in liquid phase is based in particular on measurement and correct interpretation of spin–spin coupling constants (SSCC, J) between magnetic nuclei in the molecules under investigation. This approach demands the knowledge of dependence of J-values on molecular structure parameters. Relationship between vicinal scalar constant ^3J and dihedral angle θ is most well-known and widely used in practice. For the first time the dependence $^3J = f(\theta)$ was defined and described for the case of proton–proton scalar interactions ($^3J_{HH}$) in substituted ethane's $R_1R_2HCCHR_3R_4$ by Martin Karplus [1, 2]. During a few decades the original, so-called, Karplus relationship has been widely discussed and modified many times both by the author himself [3] and by other scientists [4–21].

The main result of these theoretical discussions and experimental studies is the introduction of additional terms into the initial Karplus equation describing electronegativity and relative orientation of substituents. So-called, extended Karplus-type empirical modifications of Pachler [7–9], Altona et al. [11, 14–16, 18, 19], Gandour et al. [17] and Ōsawa et al. [20, 21] differ by the number of varied parameters and by the choice of adequate mathematical function to describe effects of substituent electronegativity and substituent orientation relative to the coupled protons. Besides, in these modifications for calculation of corresponding terms, which describe the magnitude of each substituent's effect, either Huggins atomic electronegativity [22] or group electronegativity of Mullay [23, 24] was used. At last, a lot of special modifications of the Karplus relationship were suggested and used for conformational analysis of local groups of molecules (for peptide systems [10, 13], for small rigid molecules such as steroids [5, 14, 25, 26]) and also for investigation of their separate fragments: prediction of $^3J_{HH}$ in six-membered [14, 27] and five-membered [26] saturated rings.

As empirical parameters in all local modifications are determined by fitting corresponding equation to the experimental data derived from a single-type molecular system, the applicability of such a Karplus equation to other systems has been limited in general. Moreover, for the most correct calculation of vicinal SSCCs there are some special equations, which take into account additional factors: dependences of $^3J_{HH}$ on H–C–C valence angles [20, 21, 28, 29], C–C bond length and through-space interactions between scalar coupled protons [20, 21]. Not surprisingly, the main sources of differences between experimental and calculated values of $^3J_{HH}$ are usually poor parameterization and time-averaged experimental data due to fast (in NMR time-scale) exchange between some conformers [30–37]. For example, usual root-mean square deviation (rmsd) is about 0.7–0.8 Hz for Altona [14] or Gandour [17] equations in which $^3J_{HH}$ dependences only on dihedral angle and substituent electronegativity are taken into account. In case of Osawa equation [20, 21] that contains 11 mutually independent terms and 22 adjustable parameters, rmsd is only 0.33 Hz [21]. Nevertheless, in spite of low accuracy Altona modification of the Karplus relationship is the most well-known and has been more often used in practice. First of all it is due to more simple procedure of calculation on the basis of suitable and accessible graphical PC Program “MestRe*J*” [38]. It should be noted that even in the case of spectra of strongly coupled spin systems the experimental accuracy of $^3J_{\mathrm{HH}}$ values, determined by different correlation methods of NMR spectroscopy [39] and also by usage of special data processing methods [40, 41], is usually better than the indicated rmsd-values and experimental errors are not more than 0.2 Hz.

Thus, the general approach in assessing the quality of any modification of the Karplus relationship includes a comparison of the calculated values of $^3J_{\mathrm{HH}}^{\mathrm{calc.}}$ with the corresponding experimental values of $^3J_{\mathrm{HH}}^{\mathrm{exp.}}$. The dihedral angles θ_{HH} necessary for calculating $^3J_{\mathrm{HH}}$ not always can be obtained experimentally on the basis of X-ray crystallographic data because of low accuracy in the H atom position. Only neutron diffraction studies can help solve this problem [14]. Therefore, usually θ_{HH} values are set by optimizing of molecular geometry using one of the molecular modeling methods. For this purpose time-consuming methods of molecular mechanics (MM+, MM1, MM2) were applied most often [11, 14, 21, 42]. They allow us to determine θ_{HH} enough correctly with error less than 3° [14]. Semi-empirical methods such as PM3 or AM1 may be useful for optimization of molecular geometry too [30, 42].

Therefore, the procedure of obtaining $^3J_{\mathrm{HH}}^{\mathrm{calc.}}$ on the basis of calculated values of dihedral angles $\theta_{\mathrm{HH}}^{\mathrm{calc.}}$ contains two stages: molecular structure optimization and usage of the empirical Karplus relationship. Due to different independent reasons, there are possible errors on each of these stages. They are detected only during comparison of $^3J_{\mathrm{HH}}^{\mathrm{exp.}}$ and $^3J_{\mathrm{HH}}^{\mathrm{calc.}}$, so real cause of errors cannot be established unequivocally. Obviously, you can try to compensate these errors by optimizing the parameters of the Karplus equation and using the rmsd parameter as a criterion. However, this new set of parameters will work satisfactorily only for that method of molecular geometry optimizing, from which these values of $\theta_{\mathrm{HH}}^{\mathrm{calc.}}$ were obtained.

In this research for detecting small conformational discrepancies of two molecules (**A**) and (**B**) on the base Karplus relationship it is suggested to use the comparison of experimental and calculated relative differences of vicinal SSCCs: $\Delta^3J_{\mathrm{HH}}^{\mathrm{exp.}}$ vs. $\Delta^3J_{\mathrm{HH}}^{\mathrm{calc.}}$, where $\Delta^3J_{\mathrm{HH}}^{\mathrm{exp.}} = (^3J_{\mathrm{HH}}^{(\mathbf{B})} - {}^3J_{\mathrm{HH}}^{(\mathbf{A})})^{\mathrm{exp.}}$ and $\Delta^3J_{\mathrm{HH}}^{\mathrm{calc.}} = (^3J_{\mathrm{HH}}^{(\mathbf{B})} - {}^3J_{\mathrm{HH}}^{(\mathbf{A})})^{\mathrm{calc.}}$, instead of usual comparison of absolute values of the experimental and calculated vicinal constants for each of these molecules: $(^3J_{\mathrm{HH}}^{\mathrm{exp.}}$ vs. $^3J_{\mathrm{HH}}^{\mathrm{calc.}})^{(\mathbf{A})}$ and $(^3J_{\mathrm{HH}}^{\mathrm{exp.}}$ vs. $^3J_{\mathrm{HH}}^{\mathrm{calc.}})^{(\mathbf{B})}$. Because of comparison of molecules (**A**) and (**B**), which have similar structures and in which the difference between corresponding dihedral angles is not more 10°–15°, all calculating errors of these angles $(\theta_{\mathrm{HH}}^{(\mathbf{A})\mathrm{calc.}}, \theta_{\mathrm{HH}}^{(\mathbf{B})\mathrm{calc.}})$ and of corresponding vicinal constants $(^3J_{\mathrm{HH}}^{(\mathbf{A})\mathrm{calc.}}, {}^3J_{\mathrm{HH}}^{(\mathbf{B})\mathrm{calc.}})$ in case of the same methods of structure optimization of these molecules and application of the same modified Karplus-type equation for calculation $^3J_{\mathrm{HH}}^{(\mathbf{A})}$ and $^3J_{\mathrm{HH}}^{(\mathbf{A})}$ have systematic character and must be almost the same. This approach may theoretically eliminate different inputs into errors of $\Delta^3J_{\mathrm{HH}}^{\mathrm{calc.}}$ values and increase sensitivity of $^3J_{\mathrm{HH}}$ data to conformational discrepancies of comparable molecules (**A**) and (**B**).

It should be noted that the detection and experimental evaluation of conformational features of biologically active organic molecules in solution are one of the most important problems of conformational analysis of these compounds and are necessary for predicting their useful medical properties. In particular, specific biological properties of modified steroid hormone analogues appear when small alkyl substituents are introduced into different positions of the rings A, B and C.

2 Results and Discussion

For experimental verification of the effectiveness of the use of the relative changes vicinal constants Δ^3J_{HH} to detect conformational differences of molecules were selected five rigid steroids: 19-nor-D-homotestosterone (**1**), two 3-methoxy-6-oxa-8-iso-estra-1,3,5(10)-trienes (**2**) which have different substituents in position 17 (carbonyl in (**2a**) and *β*-acetoxy in (**2b**) groups) and two 3-methoxy-6-oxa-D-homo-8-isoestra-1,3,5(10)-triene-17a-ons (**3**). One of them (**3b**) contains methyl group in position 7*β* (Fig. 1) instead proton in steroid (**3a**). The choice of conformationally rigid steroid molecules as model compounds, leads to elimination of dynamic averaging of experimental vicinal constants ${}^3J_{\mathrm{HH}}^{\mathrm{exp.}}$.

NMR spectra of these steroids in chloroform-d_1 were studied using a spectrometer DPX 300 "Bruker" and some homo- and heteronuclear correlation methods (COSY-DQF [43, 44], HSQC [45], COLOC [46], NOESY [47] and *J*-COSY [48]) and complete ^{1}H and ^{13}C signal assignments were described earlier in [49–53]. All values of geminal and vicinal SSCC ${}^{2,3}J_{\mathrm{HH}}$ were defined with high accuracy (less ± 0.2 Hz) both by using signal analysis of crowded ^{1}H spectra and interpretation of phase-sensitive cross-peak structure COSY-DQF spectra. These values of

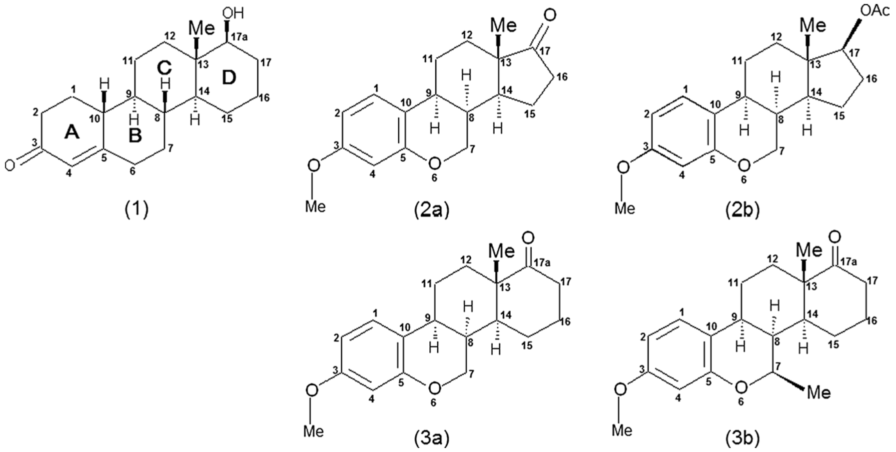

Fig. 1 Structures of model steroids (**1**)–(**3**). Rings A, B, C and D are shown on steroid (**1**) and numbers of atoms are given by figures

Table 1 Experimental and calculated (MM$^+$, MM2, PM3) vicinal SSCCs ($^3J_{HH}$, in Hz) and dihedral angles (θ_{HH}, in degrees) for 19-nor-D-homotestosterone (**1**)

No.	H_i–H_j	$^3J_{ij}^{exp.}$	$\theta_{ij}^{exp.\ b}$	$\theta_{ij}^{calc.}$			$^3J_{ij}^{calc.\ a}$		
				MM$^+$	MM2	PM3	MM$^+$	MM2	PM3
1	1α–2α	4.8	309.0	305.3	306.7	300.7	3.95	4.26	3.09
2	1α–2β	13.0	192.5	185.5	188.7	183.8	13.47	13.3	13.53
3	1α–10β	9.6	149.2	165.6	169.1	173.2	12.62	12.93	13.19
4	1β–2α	2.8	60.9	63.1	62.8	57.0	2.43	2.48	3.51
5	1β–2β	4.8	308.5	303.3	304.8	300	3.68	3.97	3.07
6	1β–10β	5.0	49.2	46.5	50.5	58.1	5.51	4.75	3.39
7	6α–7α	4.2	53.7	55.4	58.5	56.7	3.86	3.28	3.61
8	6α–7β	2.6	298.0	297.4	302.1	300.5	2.50	3.32	3.03
9	6β–7α	13.7	~ 180	173.6	175.1	172.9	13.21	13.27	13.18
10	6β–7β	5.4	47.9	55.6	58.7	56.7	3.86	3.28	3.65
11	7α–8β	11.6	162.7	179.3	179.6	178.7	12.14	12.15	12.14
12	7β–8β	3.4	301.9	299.4	298.9	294.4	2.97	2.89	2.22
13	8β–9α	12.0	183.5	182.2	182.3	181.3	12.03	12.03	12.04
14	8β–14α	10.4	203.9	181.2	181.1	183.1	12.25	12.25	12.22
15	9α–11α	3.4	58.1	55.6	56.7	61.1	3.82	3.63	2.89
16	9α–11β	10.9	156.7	173.8	173.5	176.3	12.57	12.56	12.65
17	11α–12α	3.9	304.6	304.7	304.4	302.9	3.88	3.82	3.54
18	11α–12β	3.6	56.4	61.7	59.6	58.7	2.65	2.99	3.17
19	11β–12α	13.7	~ 180	188.2	189.2	186.6	13.12	13.06	13.20
20	11β–12β	3.7	303.8	305.2	304.4	302.5	3.97	3.82	3.46
21	14α–15α	3.1	300.0	300.8	299.7	296.0	3.23	3.21	2.46
22	14α–15β	12.2	192.6	181.4	182.8	180.6	12.79	12.76	12.79
23	15α–16α	4.3	51.4	54.3	55.7	56.8	4.03	3.60	3.56
24	15α–16β	2.5	296.8	296.1	298.7	300.5	2.39	2.82	2.98
25	15β–16α	13.0	171.4	171.5	170.9	173.0	13.01	12.97	13.09
26	15β–16β	4.1	54.2	53.3	53.9	56.7	4.27	4.16	3.63
27	16α–17α	4.4	− 307.5	307.4	306.2	301.9	4.37	4.13	3.31
28	16α–17β	13.1	190.3	189.5	189.9	184.9	13.16	13.13	13.40
29	16β–17α	2.5	62.5	65.6	63.0	58.2	2.02	2.43	3.26
30	16β–17β	4.3	306.3	307.8	306.7	301.2	4.61	4.38	3.32
31	17α–17aα	4.6	54.7	55.4	55.2	61.9	4.48	4.51	3.43
32	17β–17aα	11.4	184.3	174.0	172.3	178.5	10.99	10.86	11.25

[a] Generalized Eq. (1) with β-effect was used [14]

[b] $\theta^{exp.}$ were estimated from $^3J^{exp.}$ data [49, 50] by using inverse application of Eq. (1)

experimental vicinal constants $^3J_{HH}^{exp.}$ of steroids (**1**)–(**3**) are presented in Tables 1, 2, 3, 4, 5 alongside with corresponding calculated data $^3J_{HH}^{calc.}$.

Calculation of vicinal SSCC magnitudes was made on the basis of most generalized form of Altona's modification [14] of Karplus relationship $^3J_{HH} = f(\theta_{HH})$:

Table 2 Experimental (NMR) and calculated (PM3) vicinal SSCCs ($^3J_{HH}$, in Hz) and dihedral angles (θ_{HH}, in degrees) for estrogen 8α-analogue (**2a**)

No.	H_i–H_j	$^3J^{exp.}$	$\theta^{exp.\ a}$	$\theta^{calc.}$	$^3J^{calc.}$	$^3J^{calc.} - {}^3J^{exp.}$	$\theta^{calc.} - \theta^{exp.}$
1	7β–8α	12.0	180	180.2	11.35	−0.65	0.2
2	7α–8α	3.4	61.6	57.7	4.01	0.61	−3.9
3	8α–9α	4.4	309.1	315.4	5.66	1.26	6.3
4	9α–11α	4.4	52.4	57.6	3.46	−0.94	5.2
5	9α–11β	12.2	167.8	173.5	12.56	0.36	5.7
6	11α–12α	3.7	303.6	300.2	3.08	−0.62	−3.4
7	11α–12β	2.9	60.3	57.0	3.48	0.58	−3.3
8	11β–12α	13.5	180	184.2	13.28	−0.22	4.2
9	11β–12β	3.3	301.8	301.1	3.17	−0.13	−0.7
10	8α–14α	4.8	49.8	54.2	4.01	−0.79	4.4
11	14α–15α	4.8	309.5	312.7	5.41	0.61	3.2
12	14α–15β	13.2	186.2	194.2	12.11	−1.09	8
13 (1)[b]	15α–16α	8.7	31.6	18.2	11.26	2.56	−13.3
14 (2)[b]	15α–16β	2.5	246.9	259.6	0.72	−1.78	12.7
15 (3)[b]	15β–16α	7.7	137.6	136.9	7.69	−0.01	−0.7
16 (4)[b]	15β–16β	7.6	35.8	18.2	11.1	3.5	−17.6

Full signal assignment in NMR ^{1}H spectrum of steroid (**2a**) is shown in Figure S2 (Supplementary Information)

[a] For direct ($^3J = f(\theta)$) and inverse ($\theta = f(^3J)$) calculations was used Eq. (1) [14, 37]

[b] Vicinal SSCCs in five-membered ring D have additional numbering (1, 2, 3, 4), which is used in Tables 3, 4 and in Fig. 3

Table 3 Experimental (NMR) and calculated (PM3, MM2) vicinal SSCCs ($^3J_{HH}$, in Hz) and dihedral angles θ (°) for five-membered ring D of estrogen 8α-analogues (**2a**) and (**2b**)

No.	H_i–H_j	$^3J^{exp.}$		θ_{PM3}		θ_{MM2}		$^3J^{calc.}_{PM3}$		$^3J^{calc.}_{MM2}$	
		(**2a**)	(**2b**)	(**2a**)	(**2b**)	(**2a**)	(**2b**)	(**2a**)	(**2b**)	(**2a**)	(**2b**)
1	15α–16α	8.7	9.0	18.2	11.5	25.2	10.5	11.26	11.65	10.21	11.73
2	15α–16β	2.5	3.4	259.6	253.3	262.4	252.4	0.72	1.54	0.49	1.66
3	15β–16α	7.7	5.9	136.9	129.0	146.4	131.3	7.69	5.77	9.86	6.30
4	15β–16β	7.6	8.2	18.2	11.5	23.6	13.1	11.1	11.61	10.39	11.47

Full signal assignment in NMR ^{1}H spectrum of steroid (**2b**) is shown in Figure S3 (Supplementary Information)

Table 4 Difference between experimental (NMR) and calculated (PM3) vicinal SSCCs ($^3J_{HH}$, in Hz) in ring D of estrogen 8α-analogues (**2a**) and (**2b**)

No.	H_i–H_j	$\Delta^3J^{exp.} = {}^3J^{(\mathbf{2b})} - {}^3J^{(\mathbf{2a})}$	$\Delta^3J^{calc.}_{PM3} = {}^3J^{(\mathbf{2b})} - {}^3J^{(\mathbf{2a})}$	$\Delta\Delta^3J = \Delta^3J^{calc.}_{PM3} - \Delta^3J^{exp.}$
1	15α–16α	0.3	0.39	0.09
2	15α–16β	0.9	0.82	−0.08
3	15β–16α	−1.8	−1.92	0.12
4	15β–16β	0.6	0.51	−0.09

Table 5 Calculated (PM3, MM2) and experimental dihedral angles (θ_{HH}, in degrees) and vicinal SSCCs ($^{3}J_{\mathrm{HH}}$, in Hz) for 6-oxa-D-homo-8α-analogues (**3a**) and (**3b**) of steroid estrogen

No.	H_i–H_j	$\theta^{\mathrm{calc.}}_{\mathrm{PM3}}$		$\theta^{\mathrm{calc.}}_{\mathrm{MM2}}$		$^{3}J^{\mathrm{calc.}}_{\mathrm{PM3}}$		$^{3}J^{\mathrm{calc.}}_{\mathrm{MM2}}$		$^{3}J^{\mathrm{exp.}}$	
		(**3a**)	(**3b**)	(**3a**)	(**3b**)	(**3a**)	(**3b**)	(**3a**)	(**3b**)	(**3a**)	(**3b**)
1	7α–8α	58.1	51.8	66.4	58.6	3.95	4.72	2.68	3.71	3.4	4.2
2	8α–9α	312.4	319.5	312.9	322.1	4.98	6.52	5.18	6.92	4.8	6.3
3	9α–11α	52.5	63.5	56.0	54.7	4.38	2.52	3.75	3.98	4.8	2.5[a]
4	9α–11β	177.1	180.4	172.1	170.7	12.67	12.69	12.49	12.41	12.2	12.2
5	11α–12α	299.0	295.9	302.7	300.2	2.87	2.35	3.54	3.08	3.8	3.7
6	11α–12β	55.5	52.4	58.4	56.0	3.77	4.37	3.23	3.67	3.5	3.5
7	11β–12α	182.6	180.7	187.3	185.6	13.43	13.45	13.26	13.34	13.5	13.5
8	11β–12β	299.0	295.9	303.0	301.3	2.83	2.32	3.55	3.24	4.6	4.7
9	8α–14α	50.8	40.6	53.3	42.5	4.51	6.32	4.08	5.99	4.8	6.8
10	14α–15α	288.6	286.8	293.3	291.6	1.48	1.28	2.07	1.84	2.4	2.8
11	14α–15β	171.5	169.6	175.6	173.9	12.52	12.38	12.72	12.65	12.8	12.6
12	15α–16α	58.8	56.6	55.4	53.9	3.20	3.60	3.82	4.11	4.3	4.4
13	15α–16β	302.7	300.7	298.3	297.0	3.36	3.01	2.61	2.40	3.4	3.0
14	15β–16α	176.0	173.7	171.6	170.3	13.20	13.12	13.01	12.93	13.4	13.4
15	15β–16β	59.9	57.8	54.5	53.5	3.06	3.43	4.04	4.23	4.4	4.5
16	16α–17α	308.2	309.6	308.1	309.0	4.32	4.59	4.3	4.47	4.8	4.8
17	16α–17β	191.9	193.3	191.1	192.1	13.07	12.93	13.13	13.05	13.5	13.2
18	16β–17α	64.00	65.2	65.00	65.6	2.27	2.08	2.11	2.02	2.0	2.0
19	16β–17β	307.6	308.9	308.0	308.7	4.57	4.83	4.65	4.79	6.9	6.9

Full signal assignments in NMR ^{1}H spectrum of steroids (**3a**) and (**3b**) are shown in Figures S4 and S5 (Supplementary Information), respectively

[a] This value could not determine enough correctly because of crowded signals of 8α and 9α protons

$$^{3}J(\theta) = P_1 \cos^2\theta + P_2 \cos\theta + P_3 + \sum \Delta\chi_i \left(P_4 + P_5 \cos^2\left(\xi_i\theta + P_6|\Delta\chi_i|\right)\right), \tag{1}$$

where $\Delta\chi_{\mathrm{i}}$ is the group electronegativity $\Delta\chi_{\mathrm{i}}^{\mathrm{group.}}$, which is calculated on the basis of Huggens atom substituent electronegativity [22] in α- and β-position of a substituent "i" ($i = 1, 2, 3, 4$) in ethane fragment $\mathrm{R_1R_2HCCHR_3R_4}$: $\Delta\chi_{\mathrm{i}}^{\mathrm{group.}} = \Delta E\chi_{\mathrm{i}}^{\alpha} - P_7\Delta E\chi_i^{\beta}$; ξ_i is a coefficient: $\xi_i = 1$ for the range $0° < \theta_i < 180°$ and $\xi_i = -1$ for the range $180° < \theta_i < 360°$; $\mathrm{P_1}$–$\mathrm{P_7}$ are varied empirical parameters (in this study follow initial values were used: $P_1 = 13.70$, $P_2 = -0.73$, $P_3 = 0$, $P_4 = 0.56$, $P_5 = -2.47$, $P_6 = 16.90$, $P_7 = 0.14$).

All calculations of $^{3}J_{\mathrm{HH}}^{\mathrm{calc.}}$ were made using graphical program "MestRe*J*" [38]. Some different methods (MM^{+}, MM2 and PM3) were applied to optimize molecular geometries of compounds (**1**)–(**3**) and to determine dihedral angles $\theta_{\mathrm{HH}}^{\mathrm{calc.}}$. It allowed us to compare the results of application of these methods for correct estimation of dihedral angles and relative possibilities of methods.

2.1 ^3J-Investigation of 19-nor-D-Homotestosterone (1) Spatial Structure

Previous full signal assignment [49, 50] of about 20 aliphatic protons in NMR ^{1}H spectrum of 19-nor-D-homotestosterone (**1**) suggests that only three protons 2β, 1β and 2α form ABX-type strong coupled spin-system in the range 2.1–2.5 ppm. All other signals are situated in this spectrum separately enough and only for nine protons of them signal overlapping is observed in some separate ranges: for protons 10β, 7β, 12β in the range 1.9–2.1 ppm., for protons 15α, 1α, 17β in the range 1.4–1.6 ppm. and for protons 7α, 15β, 9α in the range 0.85–1.05 ppm. (Supplementary Information, Figure S1). It allowed us to determine almost all SSCCs $^{2\text{–}4}J_{\mathrm{HH}}$ including 32 vicinal ones $^3J_{\mathrm{HH}}$ presented in Table 1 with high accuracy.

This experimental data set of $^3J_{\mathrm{ij}}^{\mathrm{exp.}}$ was very convenient for evidence on quantitative level of steroid (**1**) spatial structure based on comparison of experimental ^3J-values with calculated vicinal constants $^3J_{\mathrm{ij}}^{\mathrm{calc.}}$. Most stable conformation of steroid (**1**) was defined by molecular mechanic methods (MM$^+$, MM2) and also by semi-empirical method PM3. As a result, three sets of corresponding dihedral angles $\theta_{\mathrm{ij}}^{\mathrm{calc.}}$ were determined (Table 1) and applied as main parameter in Altona modification (Eq. **1**) of Karplus relationship. The differences between corresponding angle values which were estimated by three different methods of molecular modeling in all cases are not more than 10°.

Simultaneously, dihedral angle differences between calculated and experimental ($\theta^{\mathrm{exp.}}$) values which were estimated on the basis of experimental vicinal constants $^3J^{\mathrm{exp.}}$ by using inverse relationship $\theta^{\mathrm{exp.}}=f(^3J^{\mathrm{exp.}})$ [38]. For example, dihedral angle difference more than 10° may be detected for seven proton–proton pairs comparing $\theta^{\mathrm{exp.}}$ with calculated angles derived from structure optimization by molecular mechanic method MM$^+$. It is not surprising that all of these seven pairs relate to protons with mutual *trans*-orientation and which have dihedral angles close to 180° because namely in this range of dihedral angles Karplus relationship possesses most low sensitivity.

Calculated vicinal SSCCs $^3J^{\mathrm{calc.}}$ obtained on the basis of dihedral angles $\theta^{\mathrm{calc.}}$ due to usage of Eq. (1) are presented in Table 1 and comparison of these data sets with experimental values are shown in Fig. 2 as some different correlations.

First of all, we should pay attention to distribution in two different ranges: 2–6 and 10–13 Hz common for rigid steroid's six-membered rings ^3J-data. The first of them corresponds to axial–axial (*ax–ax*) scalar interactions and the second includes both axial-equatorial (*ax-eq*) and equatorial–equatorial (*eq–eq*) spin–spin couplings.

Comparison of the experimental data with the results of calculations of all 32 vicinal constants by MM$^+$ and PM3 methods shows that rmsd does not exceed 1.0 Hz and there is only a slight advantage of MM$^+$ (rmsd = 0.75 Hz) compared to PM3 (rmsd = 0.92 Hz). As around 180° sensitivity of Karplus relationship $^3J=f(\theta)$ is extremely low most correct information about dihedral angles θ_{HH} is contained in values of $^3J_{ax\text{-}eq}$ and $^3J_{eq\text{-}eq}$. Distribution of 20 data points belonging to only these types of vicinal SSCCs are shown in Fig. 2 (see **a′** and **b′** correlations). In this case deviations (rmsd) are less than for full data sets and equal to 0.51 and 0.82 Hz for MM$^+$ and PM3 methods, respectively.

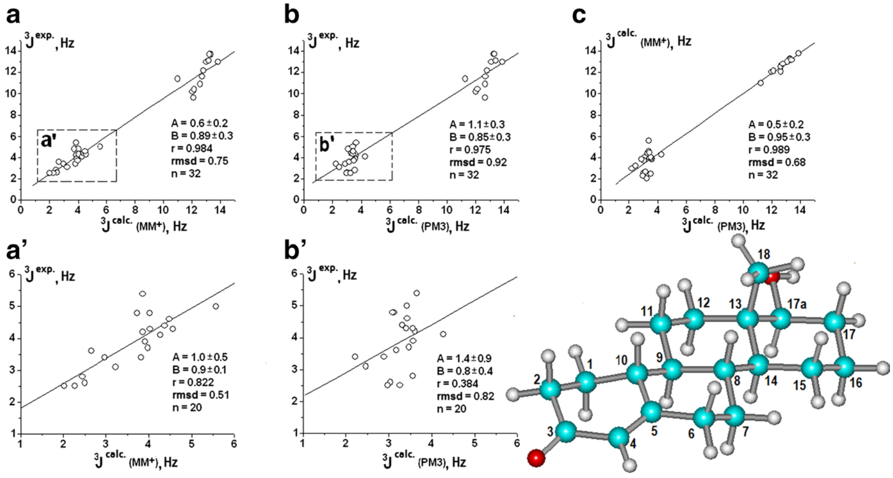

Fig. 2 Spatial structure of 19-nor-D-homotestosterone (**1**) and correlations of experimental vicinal SSCCs $^3J^{exp.}$ with calculated ones on the basis of MM$^+$ **a** and **a**′, PM3 **b** and **b**′ (**a**′ and **b**′ show only *ax-eq* and *eq–eq* scalar interactions). **c** Correlation between vicinal constants calculated on the basis of MM$^+$ and PM3 methods. Parameters of linear correlation $Y = A + BX$, r (Pearson's correlation coefficient) and rmsd (in Hz) for n (number of data points) are presented here and below

Simultaneously, a comparison of the results of using MM$^+$ and PM3 shows (Fig. 2c) a good coincidence of these data in the region of 10–13 Hz and larger spreading of data points in the region of 2–6 Hz (rmsd = 0.68 Hz).

Simultaneously, a comparison of the results of using MM$^+$ and PM3 shows (Fig. 2c) a good coincidence of these data in the region of 10–13 Hz and larger spreading of data points in the range of 2–6 Hz (rmsd = 0.68 Hz). It's clear that all dihedral angles corresponding to vicinal constants $^3J_{ax\text{-}eq}$ and $^3J_{eq\text{-}eq}$ of steroid (**1**) are located in two areas of Karplus relationship: near 60° and 300° and they differ from these values by not more than 10°–15°. Conformational analysis of the steroid (**1**) using Karplus-type relationship $^3J_{HH} = f(\theta)$ of Altona et al. [14] (1) and comparing the experimental and calculated constants is a typical example of such studies with the usual scatter of experimental and calculated data [54]. The other two examples considered in the following sections demonstrate an alternative approach based on comparing the relative values of the experimental and calculated vicinal constants in two closely related steroids.

2.2 Comparison of Spatial Structures of Estrogen 8α-Analogues (2a) and (2b)

Conformational differences of two 8α-analogues of estrogens (**2a**) and (**2b**) are the result of changing the structure of the substituent at position 17 of five-membered ring D: carbonyl group in steroid (**2a**) is replaced by β-acetoxy group in (**2b**). It leads to increasing of steric interaction in ethane fragment $H_2C^{15}–C^{16}H_2$ in (**2b**) compared to (**2a**) which is clearly visible on Newman's projections in Fig. 3. As

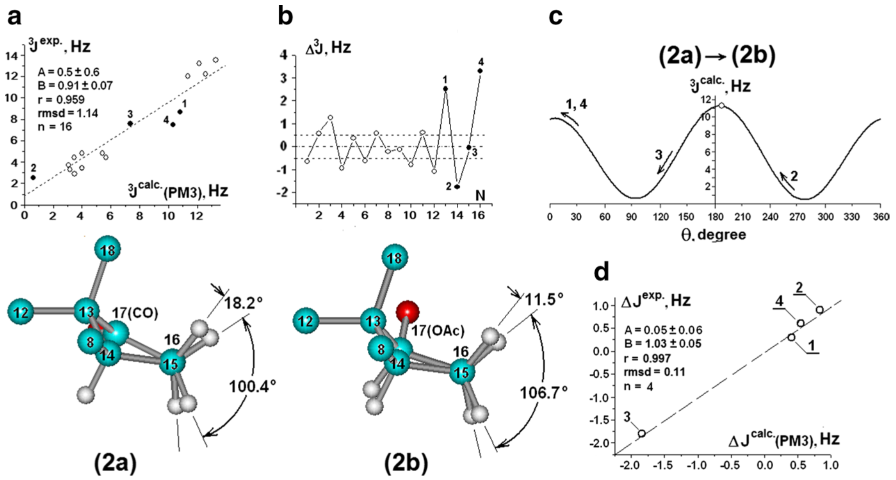

Fig. 3 Newman's projections of ethane fragment $C^{15}H_2$–$C^{16}H_2$ in five-membered ring D for steroids (**2a**) and (**2b**). **a** Comparison of experimental and calculated (PM3) vicinal SSCCs. **b** Difference between calculated and experimental ^3J-data for sixteen proton pairs in six- (open circles) and five-membered (filled circles) rings (N corresponds to the number of proton pair in Table 2). **c** Graphic of Karplus dependence on which is shown (by arrows) the sign of constant change in case of the transition from steroid (**2a**) to (**2b**). **d** Comparison of relative experimental and calculated vicinal SSCCs for coupled protons in five-membered ring D (points 1–4 correspond to proton–proton pairs in Tables 3 and 4)

a result, dihedral angles between vicinal protons in steroids (**2a**) and (**2b**) differ by several degrees. For example, according to structure optimization by semi-empirical method PM3 this difference for angle $\theta_{15\beta-16\beta}$ is 6.7° and for angle $\theta_{15\alpha-16\beta}$ is 6.3°. The same calculations of $\theta_{15\beta-16\beta}$ and $\theta_{15\alpha-16\beta}$ by method MM2 show these differences are about 10°. For steroid (**2a**) all results (including proton–proton scalar interactions in rings B and C) of experimental and calculated (by PM3 method) ways of $^3J_{\mathrm{HH}}$ and θ_{HH} determination are presented in Table 2. The comparison of $^3J^{\mathrm{exp.}}$ and $^3J^{\mathrm{calc.}}_{\mathrm{PM3}}$ is shown in Fig. 3a on which data points corresponding to four scalar interactions in five-membered ring D are marked by solid circles and have numbering 1–4 as indicated in Tables 2, 3. Scalar interactions in rings B and C in Fig. 3a, b are shown by open circles.

From these data it is clearly seen that the scatter of experimental and calculated vicinal constants for almost all proton–proton pairs in six-membered rings B and C does not exceed 1.0 Hz, while for pairs of vicinal protons it is much larger and reaches 3.5 Hz for $^3J_{15\beta-16\beta}$. This difference has a simple explanation; it is a follow-up to the use of the same Eq. (1) for estimating vicinal constants in six- and five-membered rings because this modification of Karplus relationship does not take into account the influence of valence angles HCC which are much different in cyclohexanes and cyclopentanes [28, 29]. Thus, it is impossible to prove correctly the conformational differences between steroids (**2a**) and (**2b**) by using a comparison of the absolute values of the experimental and calculated vicinal constant. To decide this problem it will be necessary to involve additional calculations by means of other modifications of the Karplus relationship $^{3}J_{\mathrm{HH}} = f(\theta_{\mathrm{HH}})$ that are more suitable for cyclopentanes.

More straightforward algorithm for solving this problem consists in using a comparison of the relative values of the experimental calculated $^{3}J_{\mathrm{HH}}$. In order to demonstrate the possibilities of this approach on qualitative level nature of changes $^{3}J_{\mathrm{HH}}$ and θ_{HH} for five-membered ring D during transition from steroid (**2a**) to (**2b**) was analyzed. The results are shown in Fig. 3c by using graphical representation of dependence $^{3}J_{\mathrm{HH}} = f(\theta_{\mathrm{HH}})$ and arrows. Such transition is accompanied by the following changes of $^{3}J_{\mathrm{HH}}$ and θ_{HH}: there is a decrease in all dihedral angles θ_{HH} within a few degrees that corresponds to the clockwise rotation of methylene protons $C^{15}H_2$ around C^{15}–C^{16} bond simultaneously vicinal SSCCs $^{3}J_{15\alpha-16\alpha}$, $^{3}J_{15\beta-16\beta}$ and $^{3}J_{15\alpha-16\beta}$ are increased and only $^{3}J_{15\beta-16\alpha}$ is decreased.

These changes of $^{3}J_{\mathrm{HH}}^{\mathrm{calc.}}$ completely correspond to a changes of their experimental values $^{3}J_{\mathrm{HH}}^{\mathrm{exp.}}$ not only on a qualitative but also a quantitative level (Table 4). Comparison of these experimental and calculated changes in case of application PM3 method of structure optimization is presented in Fig. 3d. For correlation $\Delta^{3}J^{\mathrm{exp.}}$ vs. $\Delta^{3}J^{\mathrm{calc.}}$, where $\Delta^{3}J = {}^{3}J^{(\mathbf{2b})} - {}^{3}J^{(\mathbf{2a})}$, parameter rmsd is equal to 0.11 Hz in spite of very large discrepancies between the absolute values of the calculated and experimental constants $^{3}J_{\mathrm{HH}}$ in five-membered ring D of steroids (**2a**) and (**2b**). Of cause this result does not take into account all other data points for six-membered rings B and C, which are located near zero point and sensitive to random errors in the measurement of experimental vicinal SSCCs. It should be noted that the similar usage of the calculation method MM2 gave less impressive results at the quantitative level (Table 3) compared with data obtained on the basis of the semi-empirical method PM3. Nevertheless, these results quite objectively reflect the conformational differences of the considered steroids at a qualitative level.

2.3 Comparison of Spatial Structures of Estrogen 8α-Analogues (3a) and (3b)

Structures of D-homo-8α-analogues of estrogens (**3a**) and (**3b**) differ from each other by only methyl group in position 7β in (**3b**) instead of proton in (**3a**). As a result of the appearance in the 7β position of the methyl group with the axial orientation between the rings B and C of the steroid (**3b**) an increase of the steric interactions between 7β- and 13β-methyl groups in the β-area of this molecule should occur as compared with the steroid (**3a**). This, in its turn, should lead to a change in the mutual spatial location of the bridge protons 8α, 9α and 14α (Fig. 4).

According to preliminary calculations of preferred spatial structures of both steroids sufficiently large differences of dihedral angles $\theta_{7\alpha-8\alpha}$, $\theta_{8\alpha-9\alpha}$ and $\theta_{8\alpha-14\alpha}$ are observed which are shown by arrows. These and other dihedral angles were defined on the basis of structure optimization by using of semi-empirical (PM3) and force field (MM2) methods and then Karplus-type Eq. (1) was used to determine appropriate vicinal SSCCs $^{3}J_{\mathrm{HH}}^{\mathrm{calc.}}$. All results for molecules (**3a**) and (**3b**) are collected in Table 5.

Determination of experimental values of $^{3}J_{\mathrm{HH}}^{\mathrm{exp.}}$ caused the greatest difficulties in case of steroid (**3b**) NMR-analysis. Since the accuracy of calculating the absolute

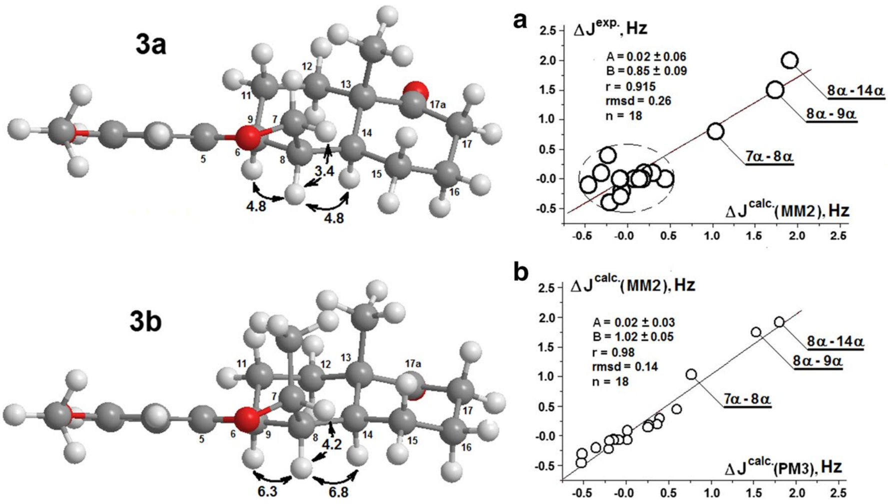

Fig. 4 Spatial structures of steroids (**3a**) and (**3b**) on which angles θ_{HH} most sensitive to 7β-substituent are shown by arrows and the values (in Hz) of ${}^3J_{HH}^{exp.}$ are given by figures. **a** Correlation of relative experimental and calculated by MM2 method vicinal SSCCs. **b** Correlations of relative vicinal SSCCs defined by PM3 and MM2 calculated methods

values of the calculated vicinal constants is not high, their comparison with the experimental data [as well as in the case of comparative study of steroids (**2a**) and (**2b**)] was carried out by comparing relative, and not absolute values, the difference in the ${}^3J_{HH}$ constants considered steroids: $\Delta^3J_{ij} = {}^3J_{ij}^{(3b)} - {}^3J_{ij}^{(3a)}$. The correlation between the calculated and experimental values of vicinal SSCC $\Delta^3J_{ij}^{exp.}$ vs. $\Delta^3J_{ij}^{calc.}$ for 18 of the 19 pairs of protons specified in Table 5 is shown in Fig. 4a. From the complete set of data one pair of 9α–11α was excluded, for which the exact value of ${}^3J_{9\alpha-11\alpha}$ for a steroid (**3b**) could not be determined sufficiently enough because of crowded signals of 8α and 9α protons.

It is clearly seen that for steroids (**3a**) and (**3b**) the strongest difference in experimental constants ($\Delta^3J_{HH}^{exp.}$) is observed for the proton–proton pairs 7α–8α, 8α–9α and 8α–14α. This is in full accordance with the predicted changes in their calculated values ($\Delta^3J_{HH}^{calc.}$) based on the MM2 and PM3 methods. For example, the correlation between the experimental and calculated data obtained by the MM2 method $\Delta^3J_{HH}^{exp.}$ vs. $\Delta^3J_{HH}^{MM2}$ is characterized by the rmsd parameter equal to 0.26 Hz (Fig. 4a) and the spread of the remaining 15 points in the range ± 0.5 Hz (marked by an ellipsoid on the graph) is completely explained by random errors in the experimental determination of vicinal constants. The last statement is partially confirmed by a sufficiently good correlation (rmsd = 0.14 Hz) of the calculating methods among themselves (Fig. 4b), including the region ± 0.5 Hz.

It should be noted that the experimental values of ${}^3J_{ij}^{exp.}$ for steroids (**3a**) and (**3b**) were determined with the greatest possible accuracy by comparing the results of several experiments (DQF-COSY, *J*-COSY, HSQCnd) and involving proton spectra simulation of individual spin systems containing 7–9 nuclei. In Fig. 5.1 the

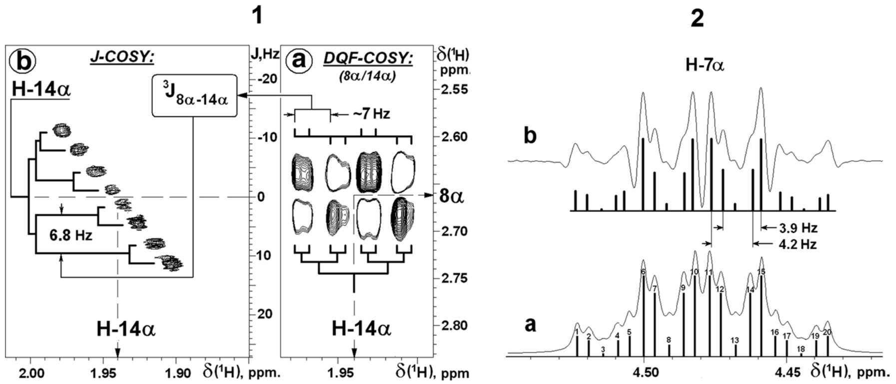

Fig. 5 **1** Fragments and analysis of **a** DQF-COSY and **b** *J*-COSY spectra to define correct value of vicinal constant $^3J_{8\alpha\text{–}14\alpha}$ for steroid (**3b**). **2** Fragment and analysis of **a** simulated and **b** experimental spectrum NMR ^{1}H of proton 7α to determine correct value of $^3J_{7\alpha\text{–}8\alpha}$ for steroid (**3b**)

procedure for determining the vicinal constant $^3J_{8\alpha\text{–}14\alpha}$ in a steroid (**3b**) using the analysis of the multiple structure of the cross-peak 8α/14α in the DQF-COSY spectrum and the projection to the F1 axis of the proton signal 14α in the spectrum *J*-COSY is shown as an example of the combined usage of several two-dimensional NMR methods.

Each of these spectra shows that the proton signal 14α at 1.94 ppm. is a doublet of the doublet of doublets (ddd), but only the *anti*-phase structure of the cross-peak 8α/14α in the DQF-COSY spectrum gives an unambiguous indication that the average constant (~ 7 Hz) belongs to the scalar interaction between protons 8α and 14α.

However, in the DQF-COSY spectrum due to the relatively low digital resolution the accuracy of constant determination by measuring the splitting value between the *anti*-phase components of the cross peak is small (~ ± 0.5 Hz). Moreover, the measured value in this case is, as a rule, overestimated in comparison with the actual value of $^3J_{8\alpha\text{–}14\alpha}$. Therefore, a more accurate value of $^3J_{8\alpha\text{–}14\alpha}$ is equal to (6.8 ± 0.1) Hz was obtained from the analysis in the *J*-COSY spectrum of the projections of components of the same signal on the F1 axis, which correspond to the values of the scalar interaction constants.

As another example the definition of the vicinal constant $^3J_{7\alpha\text{–}8\alpha}$ from the analysis of the multiple structure of the proton signal 7α in the ^{1}H NMR spectrum of the steroid (**3b**), which, due to superposition of the signals of the protons 8α and 9α in the range 2.55–2.70 ppm, has complicated multiple structure, is shown in Fig. 5.2. According to the spectral simulation data, in addition to the lines corresponding to the first-order spectrum, depending on the ratio between the constants $^3J_{8\alpha\text{–}9\alpha}$, $^3J_{8\alpha\text{–}7\alpha}$, $^4J_{7\alpha\text{–}9\alpha}$ and the difference of the chemical shifts $\Delta\delta_{8\alpha,9\alpha} = \delta_{8\alpha} - \delta_{9\alpha}$, this signal can contain a different number of additional so-called combination lines.

Therefore, in order to determine the exact value of the vicinal constant $^3J_{7\alpha\text{–}8\alpha}$, all components of the multiple structure of the signal 7α need to be fully identified, including combinational lines (see lines 3, 8, 13 and 18). As a result of the comparison of the calculated (Fig. 5.2a) and experimental (Fig. 5.2b) spectra, the exact value of the constant $^3J_{7\alpha-8\alpha} = (4.2 \pm 0.1)$ Hz was obtained.

3 Conclusion

Five rigid steroid molecules were investigated by different 1D and 2D methods of NMR spectroscopy to obtain the most complete sets of experimental values for proton–proton vicinal SSCCs $^3J_{\mathrm{HH}}^{\mathrm{exp.}}$ with high accuracy which are necessary for conformational analysis of these steroids. These sets included a sufficiently large number of vicinal constants (from 16 to 32) measured with an accuracy of at least ± 0.2 Hz. They were applied to be compared with the calculated values of these constants $^3J_{\mathrm{HH}}^{\mathrm{calc.}}$ obtained as a result of using one of the most well-known and often used in practice the modification of empirical Karplus-type relationship $^3J_{\mathrm{HH}}^{\mathrm{calc.}} = f(\theta_{\mathrm{HH}}^{\mathrm{calc.}})$ of Altona et al. [14]. This modification successfully combines the convenience of use and the possibility of taking into account the electronegativities of the substituents located in the *α*- and *β*-positions of the ethane fragment. To optimize the geometry of the steroids under investigation for the subsequent determination of the necessary sets of dihedral angle ($\theta_{\mathrm{HH}}^{\mathrm{calc.}}$) values the simplest methods of molecular modeling were used such as the semi-empirical method of PM3 and the methods of molecular mechanics MM^+ and MM2.

Unfortunately, usual approach based on a comparison of the experimental and calculated values of the vicinal SSCCs $^3J_{\mathrm{HH}}$ provides only qualitative confirmation of the spatial structure of the studied steroids, since in this case a rather large spread of experimental and calculated $^3J_{\mathrm{HH}}$ is observed (rmsd is about 0.8 Hz). This corresponds to an error in estimating the dihedral angle θ_{HH} approximately within 10°–15°. Consequently, using such an algorithm, relatively small differences in the spatial structure of steroid molecules cannot be detected and studied at a quantitative level.

As an alternative approach for quantitative description by NMR of small differences in the spatial structure of rigid steroid molecules, in this research it was suggested to use the comparison of relative changes in experimental and calculated vicinal SSCCs Δ^3J_{HH} obtained for two molecules relatively similar in structure. The advantages of this alternative research method are revealed by the examples of comparative study of two pairs [(**2a**), (**2b**) and (**3a**), (**3b**)] having only small structural differences 8*α*- and D-homo-8*α*-analogues of estrogens. It was shown that a comparison of the relative changes in the experimental and calculated vicinal constants ($\Delta^3J_{\mathrm{HH}}^{\mathrm{exp.}}$ vs. $\Delta^3J_{\mathrm{HH}}^{\mathrm{calc.}}$) has a number of notable advantages.

This approach does not require additional spectral measurements since it is based on the change in the calculation algorithm and on the profound assumption that the errors introduced into the final result by any of the methods for optimizing the geometry of molecules and the modified version of the Karplus-type empirical equation are practically nearby the same for two molecules under investigation), which have only small conformational differences of dihedral angles θ_{HH} within not more than

15°. Consequently, the nature of these errors is systematic and their contribution to the calculated relative values of the vicinal constants $\Delta J_{\mathrm{HH}}^{\mathrm{calc.}}$ should be eliminated.

In addition, a comparison of the relative values of the experimental and calculated vicinal constants makes it possible not to impose strict requirements on the choice of a modification of the Karplus-type equation or to a method for optimizing the geometry of molecules, since all discrepancies between the results of applying these modifications and calculation methods will appear in absolute values of ${}^{3}J_{\mathrm{HH}}^{\mathrm{calc.}}$ and $\theta_{\mathrm{HH}}^{\mathrm{calc.}}$. The last statement has been experimentally confirmed in the present study in the comparative study of steroids (**2a**) and (**2b**) since a modification of the Karplus Eq. (1) was used to analyze the five-membered ring, the parameters of which are optimized for the study of six-membered rings. As a result, the absolute values of the design constants ${}^{3}J_{15\alpha-16\alpha}^{\mathrm{calc.}}$ and ${}^{3}J_{15\beta-16\beta}^{\mathrm{calc.}}$ for the steroid (**2a**) were overestimated compared to the experimental values of 2.56 and 3.5 Hz, respectively. At the same time, when comparing steroids (**2a**) and (**2b**), the difference between the relative experimental and calculated values of the same constants was less than 0.1 Hz (see Table 4).

The correlation between the relative changes in the calculated vicinal constants for steroids (**3a**) and (**3b**) obtained by two different PM3 and MM2 methods turned out to be unexpectedly good and encouraging (Fig. 4b). This indicates that the discrepancy between the results of using different calculation methods does not exceed the experimental error of the SSCC measurements (~ 0.2 Hz).

The proposed method for analyzing ${}^{3}J$-data on the basis of a comparison of the relative changes in the experimental and calculated vicinal constants requires further study. It is likely to be most convenient practical method in the study of series of compounds having small spatial differences due to changes in substituents at different positions of the molecules studied. In its turn, small changes in spatial structure can lead to a selective change in the useful properties of molecules in solution, including biological activity.

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