2D-NMR-Guided Constitutional Analysis of Organic Compounds Employing the Computer Program COCON[+]

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The computer program COCON is introduced as a tool for the comprehensive structure elucidation of unknown organic compounds. In particular, structural proposals made on the basis of the molecular formula and of 2D-NMR experiments can be analyzed for the existence of alternative constitutions being in agreement with the same data set. The computational speed grounds on the evaluation of ambiguous long-range connectivity information during the process of structure generation. The data set experimentally obtained for the marine natural product oroidin (**1**) was selected, because proton-poor compounds usually cause uncertainties in NMR-based structure determinations. The calculation results encourage to move from the experience-based analysis of NMR chemical shifts or of MS fragmentations to the automated evaluation of routinely available connectivity information.

The structure elucidation of unknown compounds is a central field of organic chemistry. The introduction of high-field NMR spectrometers with pulsed field gradients and sufficient sensitivity has enabled chemists to apply sophisticated 2D-NMR techniques on a routine basis. A computer-assisted method of structure elucidation[1,2] has to generate all constitutions compatible with any given information[3] and, if possible, within a time period shorter than necessary to obtain the experimental data themselves. In contrast to the constitutions of proton-rich compounds, those of proton-poor compounds are expected to be underdetermined even on the basis of connectivity information obtained from two-dimensional HMBC[4] and 1,1-ADEQUATE[5] experiments. In this paper, we describe the computer program COCON ("Constitutions from Connectivities")[6] and its application to the analysis of the experimentally obtained data set of the alkaloid oroidin (**1**)[7] isolated from the marine sponge *Agelas clathrodes.* Of course, COCON is not able to directly read connectivity data from an NMR spectrum which would be the task of a specialized peak picking process. Instead, the input file has a table format listing correlations between individually numbered heteroatoms (see the Supplementary Material of this article).

Two different COCON calculations were performed of which the first, for reasons of illustration, used exactly the hybridization states present in **1**. The second, more sophisticated analysis included the automated generation of all hybridization-state combinations being in accordance with the degrees of protonation of every atom and the ^{13}C-NMR chemical shifts (rules of interpretation see below) of **1**.

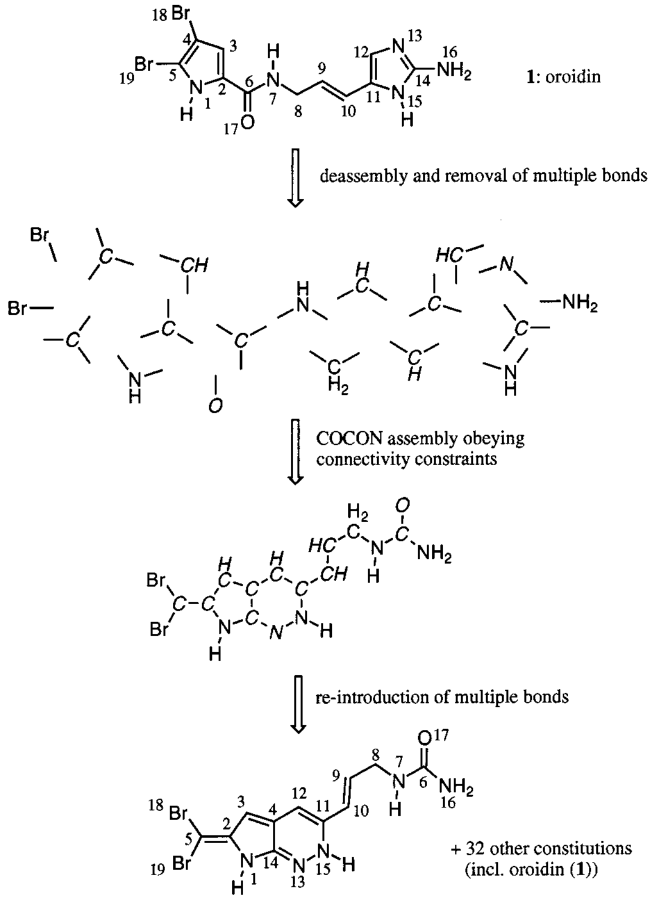

Figure 1. The search for structural alternatives with identical hybridization states: deassembly of oroidin (**1**) and connectivity-based (see Figure 2) reassembly of its fragments

Calculations assuming fixed hybridization states will be especially useful, if constitutional proposals are to be investigated for the existence of alternatives with the identical hybridization state combination. The user has then first to disassemble his own constitutional proposal. Disassembling

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[+] A basic version and the complete documentation of the software can be ordered from the corresponding authors. Recently, an internet version of the software has been installed: http://cocon.org.chemie.uni-frankfurt.de

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the constitution **1** led to 19 heavy atoms with defined hybridization states and numbers of hydrogen substituents. The computer program COCON now systematically reassembled these fragments obeying all connectivity constraints defined by the original elucidation problem (Figure 1). Finally, every generated assembly was examined for its compatibility with at least one multiple bond system corresponding to the fixed hybridization states of the atoms.[8] For oroidin (**1**), the connectivity information was derived from the four 2D-NMR experiments ^{1}H,^{1}H COSY, ^{1}H,^{13}C HSQC, ^{1}H,^{13}C HMBC, and 1,1-ADEQUATE.[9] The COCON analysis of oroidin (**1**) led to 32 alternatives of the original structure, even though the NMR analysis of the molecule had been performed as completely as possible. There were a few additional constraints applied regarding the interpretation of ^{13}C-NMR chemical shifts.[10] Cyclopropenoid and cyclobutadienoid substructures were excluded. The calculation time was 9 seconds (Indigo R4000 workstation, 100 MHz).

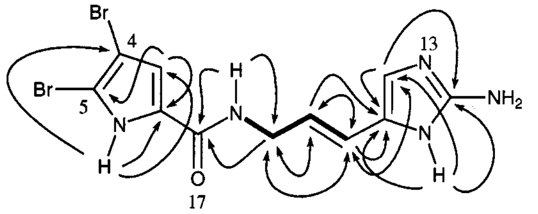

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Figure 2. 23-HMBC correlations (shown from protons to carbon atoms) were experimentally observed for oroidin (**1**); the double-headed arrows indicate HMBC correlations in both directions; the bold bonds are derived from COSY correlations; in an additional 1,1-ADEQUATE experiment, every theoretically possible correlation was observed

Of course, some of the proposed constitutions (see Figure 3) are chemically unstable and can be excluded. Nevertheless, all 33 structures must be taken into consideration, because they are in accordance with the entirety of NMR-derived connectivity information and with the molecular formula of oroidin (**1**).

In practice, the hybridization states of the involved heavy atoms may not always be unambigously clear, e. g., ^{13}C-NMR signals between $\delta = 75$ and $\delta = 115$ may correspond to sp-, sp^2-, or sp^3-hybridized carbon atoms. In our second, more in-depth calculation, the hybridization states of the involved atoms were not specified, but automatically generated by COCON according to the known degrees of protonation and ^{13}C-NMR chemical-shift rules.[11] 3008 hybridization-state combinations were generated of which, surprisingly, only 35 (1.2%) yielded constitutions at all after connectivity evaluation (16888 consitutions in total, see Figure 4). The calculation time was 28 min (SGI R10000 workstation, 195 MHz). The highest number of constitutions (4897) was calculated for the hybridization state combination #621 which differed from #879 (of oroidin) by the presence of sp^3 instead of sp^2 hybridizations for C-4 ($\delta = 98$), C-5 ($\delta = 105$), N-13, and O-17 (see Figure 2).

The narrow distribution of the constitutions over only a few hybridization-state combinations indicates that the discussion of hybridization state combinations remains very restricted as soon as good-quality connectivity information is regarded. For the given example, the IR absorption band for oroidin[7] ($\tilde{\nu} = 1655$ cm^{-1}) implies the presence of at least one carbonyl group and thereby assigns the hybridization of the single oxygen atom present to be sp^2. As a consequence, e. g. the dominant hybridization-state combination #621 leading to 29% of the 16888 calculated constitutions can be excluded (among others).

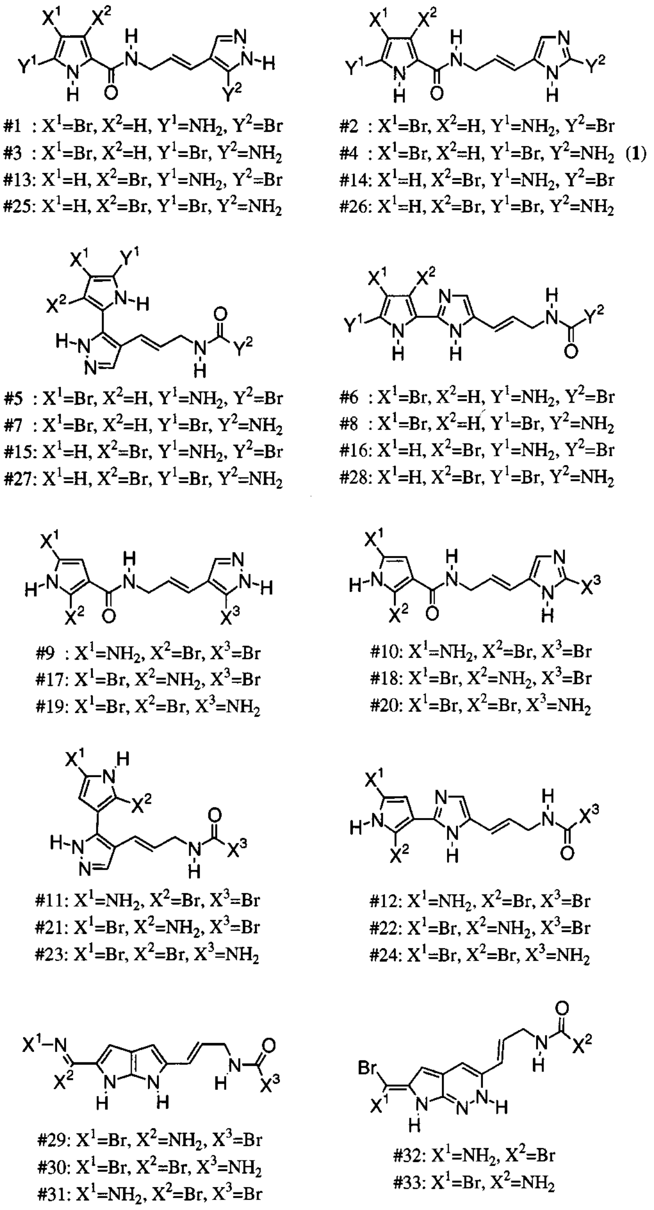

Figure 3. 33 alternative constitutions obtained on the basis of the 2D-NMR data set of oroidin (**1**); the solution numbers are marked (#) and grouped on the basis of structural similarity; all of the proposals have identical molecular formulas and hybridization-state combinations [exclusion of bonds between (a) protonated heavy atoms not showing a ^{1}H,^{1}H-COSY correlation and (b) carbon atoms of which at least one is protonated and of which none shows a 1,1-ADEQUATE correlation to the other]

Structure analysis relies on comprehensive structure generation. In addition, the calculation speed should be within the range of the time needed to perform the experiments

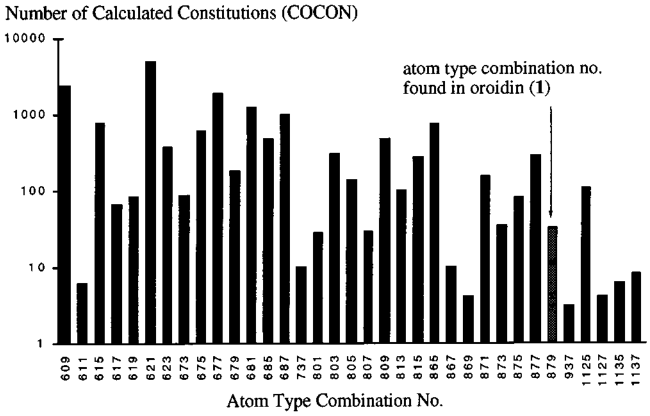

Figure 4. Distribution of in total 16888 compatible constitutions over only 35 of originally calculated 3008 hybridization-state (atom-type) combinations; the hybridization-state combination #879 is found in oroidin (**1**)

being the basis of the input data. In the following, it is briefly discussed how comprehensiveness and calculation speed of the computer program COCON are achieved. An overview of its general organization is given in Figure 5. In the input file, the order number, the degree of protonation, and the ^{13}C-NMR chemical shift of every heavy atom are defined, as well as the connectivity relations between the atoms. The heart of the COCON computer program is a structure generator examining incomplete substructures during the generation process ("permanent examinations"). "Appended examinations" take place after a complete constitution has been generated.

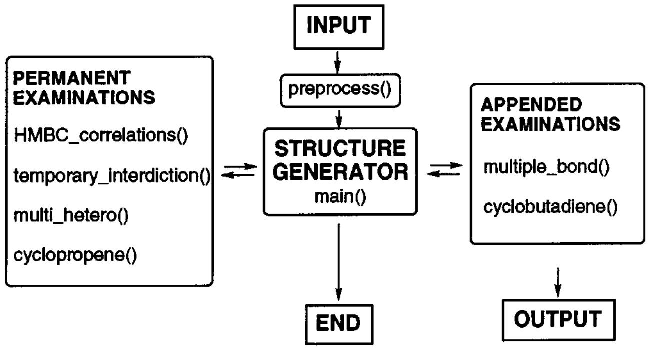

Figure 5. The structure generation by the computer program COCON is controlled by "permanent examinations" and by "appended examinations"; as soon as inconsistencies of a generated substructure with the required connectivities or other criteria are detected by the "permanent examinations", the structure generator itself jumps to the next substructure of the same size; if the discussed substructure is in agreement with all required data, it is enlarged by usually one bond and checked again; when all required atoms have been built in, "appended examinations" are carried out and the constitution may eventually be accepted as output; the program terminates if every possible combination of direct neighbors of at least one atom has been fully examined

Preprocessing (function "preprocess", Figure 5) includes the determination of general parameters such as the total number of bonds to be formed. The complex 2D-NMR-derived information is simplified by reduction to its geometrical meaning. Optional, specific inclusion or exclusion of bonds will strongly reduce the space of constitutions. The exclusion of specific bonds is taken into account on a case-to-case basis on the level of incomplete constitutions. Fixed bonds may result from the definition of substructures as well as from NMR-derived connectivity data (^{1}H,^{1}H COSY, 1,1-ADEQUATE). The pre-evaluation of COSY and of 1,1-ADEQUATE data may optionally demand the absence of bonds, as soon as bonds between atoms not showing COSY or 1,1-ADEQUATE correlations are forbidden. For COSY correlations this rule can be extended from proton-bearing carbon atoms to all proton-bearing atoms.[12] The exclusion (but not the inclusion!) of certain bonds can also be the consequence of very coarsely chosen ^{13}C-NMR chemical-shift rules.[9] It proved to be much more economical to perform detailed ^{13}C-NMR chemical-shift calculations for each completed solution subsequent to a COCON calculation, e. g. using the computer program SpecEdit.[6b] *Spectral information should not be taken into account if there is uncertainty about its interpretation.*

A detailed description of the structure generation is given in the Supporting Information of this article. An assembling algorithm has to use input information on the level of incomplete constitutions whenever it is possible. A key property of the COCON algorithm is the integrated evaluation of ambiguous connectivity information derived from HMBC spectra (function hmbc_correlations, Figures 5, 6). The sphere of a core atom consists of its direct neighbours (first sphere) and of the first spheres of these neighbours (second sphere segments). On the level of incomplete constitutions, COCON analyzes the spheres of every atom distinguishing the following three cases. If the maximal size of the total free sphere[13] around the atom is smaller than the number of the still unfulfilled HMBC correlations of the core atom (case 1, Figure 6), COCON will not accept the partial constitution. If an atom possesses a completed first sphere and if the number of free places in its second sphere is at least equal to the number of the still unfulfilled HMBC correlations of the core atom, two cases are possible: If the free positions lie within two different segments of the second sphere and if their number exactly equals the number of the unfulfilled HMBC correlations of the core atom (case 2, Figure 6), only connections between its first-sphere partners and the atoms given by the required HMBC correlations will be accepted (function temporary_interdiction, Figure 5). If exactly one second sphere has free positions (case 3, Figure 6), the bonds complying with the still unfulfilled HMBC correlations of the core atom are immediately formed. If none of the three cases is true, no action will be taken and the partial constitution is accepted.

Other permanent examinations (see Figure 5) include the check for the presence of geminal diols (function multi_hetero) which may occur in organic compounds, but may nevertheless be optionally excluded. The same is possible for cyclopropenoid structures (function cyclopropene). COCON calculates every possible multiple-bond distribution and therefore provides every mesomeric form possible for a calculated constitution. Among COCON's optional functions, the check for usually unstable cyclobutadienoid substructures (function cyclobutadiene, Figure 5) relies on the

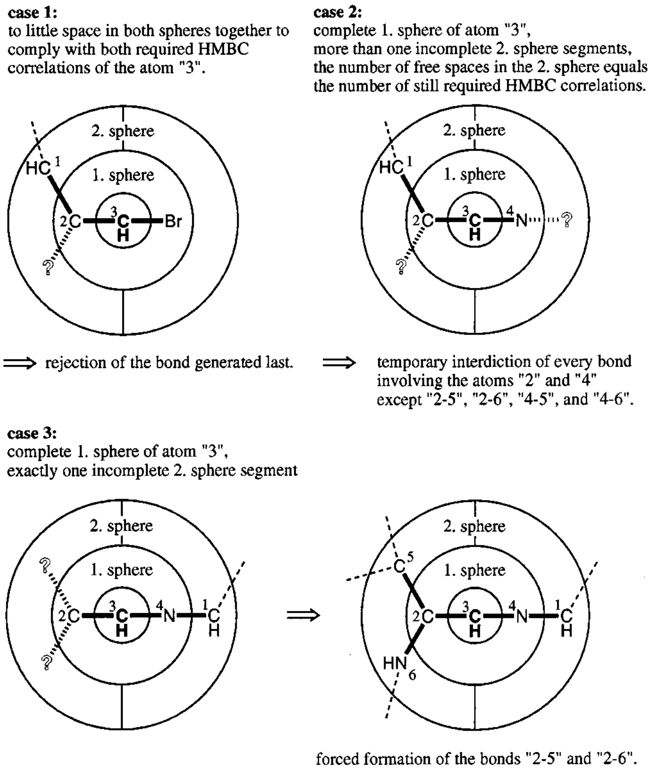

Figure 6. The three sphere constellations examined by COCON's permanent function "HMBC_correlations"; HMBC correlations between the atom "3" and the atoms "1", "5", and "6" be assumed

exhaustive consideration of all multiple-bond systems possible for a certain structure.

In summary, the computer program COCON enables organic chemists to automatically and comprehensively analyze their structural proposals for the possibility of alternative proposals being in agreement with the molecular formula and all 2D-NMR spectral data. The natural product oroidin (**1**) was chosen as a typical proton-poor alkaloid. The existence of 32 alternatives to the correct structure with identical hybridization states of the involved atoms strongly indicates the need for automated analysis of molecular constitutions. The consideration of HMBC correlations on the level of incomplete constitutions is the key to satisfying calculation times making COCON a valuable tool for routine applications. For the field of automated structure elucidation, the COCON calculation results encourage to move from the experience-based analysis of NMR chemical shifts or of MS fragmentations to the automated evaluation of routinely available connectivity information. If 2D-NMR data are available, very wide ^{13}C-NMR chemical-shift rules are sufficient to reduce the space of compatible constitutions.

Experimental Section

General: Melting points were measured with a Reichert hotstage and are uncorrected. − Nuclear magnetic resonance spectra were taken with a Bruker DRX600 spectrometer. All measurements were carried out at 300 K. − Mass spectra were measured with a Varian MAT-311 A mass spectrometer at 70 eV. − Solvents were purified and dried according to standard procedures.[14] Sephadex LH20 (Pharmacia) was used for size-exclusion chromatography. Silica gel 60 (230−400 mesh, Merck) was used for normal-phase column chromatography.

Isolation of Oroidin (1): A freeze-dried sample (31.5 g) of *Agelas clathrodes* was extracted three times with methanol/dichloromethane (2:1, 600 mL). The crude extract (8.5 g) was partitioned between *n*-hexane (300 mL) and methanol (150 mL). The methanol extract (7.8 g) was partitioned again between *n*-butanol (150 mL) and water (150 mL). The *n*-butanol phase was concentrated and the residue (2.4 g) was purified by gel chromatography (Sephadex LH-20, methanol). The oroidin-containing fractions (340 mg) were combined and fractionated by gravity column chromatography (silica, chloroform/methanol/sat. ammonia, 40:20:1). The collected oroidin (**1**) was stirred with dichloromethane (5 mL) and the insoluble part was collected yielding a light brown solid (110 mg, 0.35% of the dry weight), m.p. 230°C (dec.). − ^{1}H NMR ($[D_6]$DMSO): δ = 3.94 (dd, J = 5.5, 4.9 Hz, 2 H), 6.13 (dt, J = 16.4, 4.9 Hz, 1 H), 6.20 (d, J = 16.4 Hz, 1 H), 6.88 (s, 1 H), 6.98 (s, 1 H), 7.45 (s, 2 H, NH_2), 8.60 (t, J = 5.5 Hz, 1 H, NH), 11.95 (br s, 1 H, NH), 12.63 (br. s, 1 H, NH), 12.79 (s, 1 H, NH). − ^{13}C NMR ($[D_6]$DMSO): δ = 39.8 (CH_2), 98.0 (C), 104.6 (C), 111.1 (CH), 113.2 (CH), 116.4 (CH), 124.9 (C), 126.9 (CH), 128.2 (C), 147.8 (C), 158.8 (C). − MS (pos. FAB); *m/z* (%): 392/390/388 $[M + H]^+$ (34/72/36).

2D-NMR Experiments: The 2D-NMR data were obtained from two samples of oroidin (**1**) in $[D_6]$DMSO (49.9 mg in 0.45 mL) and in [D]chloroform/$[D_6]$DMSO (3:2, 49.6 mg of **1** in 0.50 mL). All 2D-NMR spectra were recorded with a spectral width of 14.991 ppm in F_2 (for the COSY spectrum in F_1 and F_2) and with 2048 data points (HSQC, 1,1-ADEQUATE; acquisition time of 114 ms) or with 4096 data points (COSY and HMBC; acquisition time of 228 ms). The HMBC experiments were run without low-pass J filter. The HSQC, and HMBC experiments were run with pulsed field gradients for coherence order selection. The COSY, HSQC, and HMBC experiments were run with standard pulse programs from the Bruker library. The spectra were apodizated with a squared $\pi/2$-shifted sine bell in both dimensions (except COSY with $\pi/3$ and HMBC with $\pi/4$ in F_2). The spectra were processed in F_2 with 2048 data points (HSQC, 1,1-ADEQUATE) or with 4096 data points (COSY, HMBC). (a) COSY: Relaxation delay $D1$ = 1.6 s, 2 acquisitions, 512 increments. The spectrum was processed with 4096 data points in F_2 and with 1024 in F_1 (measuring time, 1 h 1 min). (b) ^{1}H,^{13}C HSQC: Relaxation delay $D1$ = 1.8 s, delay for the evolution of $^1J_{CH}$ $D4$ = 80 ms, sweep width in F_1 of 130 ppm, 2 acquisitions, 512 increments. The spectrum was processed with 1024 data points in F_1 (measuring time, 30 min). (c) ^{1}H,^{13}C HMBC spectrum without low-pass J filter (standard pulse program from the Bruker library): relaxation delay $D1$ = 1.6 s, delay for the evolution of $^nJ_{CH}$ $D6$ = 80 ms, sweep width in F_1 of 130 ppm, 4 acquisitions, 512 increments. The spectrum was processed with 1024 data points in F_1 (measuring time, 1 h 6 min). (d) 1,1-ADEQUATE: Relaxation delay $D1$ = 2.0 s, delay for the evolution of $^1J_{CH}$ $D4$ = 1.786 ms ($D24$ = 1.190 ms), delay for the evolution of $^1J_{CC}$ $D12$ = 5.0 ms, sweep width in F_1 of 130 ppm, 96 acquisitions, 192 increments. The spectrum was processed with 256 data points in F_1 (measuring time, 15 h 10 min).

COCON Calculations: All COCON calculations used the following connectivity data: (a) direct connections as derived from the ^{1}H,^{1}H-COSY experiment: N-7 and C-8; C-8 and C-9; C-9 and C-10; (b) ambiguous two- or three-bond distances as derived from the ^{1}H,^{13}C

HMBC experiment: N-1 and C-2, C-3, C-4; C-3 and C-2, C-5; N-7 and C-6, C-8; C-8 and C-6, C-9, C-10; C-9 and C-8, C-10, C-11; C-10 and C-8, C-9, C-11, C-12; C-12 and C-10, C-11, C-14; N-15 and C-11, C-12, C-14; (c) direct connections as derived from the 1,1-ADEQUATE experiment: C-3 and C-2, C-4; C-8 and C-9; C-9 and C-8, C-10; C-10 and C-9, C-11; C-12 and C-11. The following modes of connectivity interpretation were chosen: (a) exclusion of bonds between protonated heavy atoms not showing a ^{1}H,^{1}H-COSY correlation; (b) exclusion of bonds between carbon atoms of which at least one was protonated and of which none showed a 1,1-ADEQUATE correlation to the other. For the COCON calculations with fixed hybridization states the following atom-type combination was used: N-1: >N−; C-2: >C= ($\delta = 128$); C-3: −CH= ($d = 113$); C-4: >C= ($\delta = 98$); C-5: >C= ($\delta = 105$); C-6: >C= ($\delta = 159$); N-7: >NH−; C-8: −CH$_2$− ($\delta = 40$); C-9: −CH= ($\delta = 127$); C-10: −CH= ($\delta = 116$); C-11: >C= ($\delta = 125$); C-12: >C= ($\delta = 111$); N-13: =N−; C-14: >C= ($\delta = 148$); N-15: >N−; N-16: −NH$_2$; O-17: =O; Br-18: −Br; Br-19: −Br. The COCON calculation with undefined atom-type combinations was based on the degrees of protonation of each atom, as derived from ^{1}H-NMR and DEPT spectra.

Acknowledgments

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[8] It is not always possible to introduce a multiple-bond system into every generated geometry. E. g., it is impossible to introduce three double bonds into the skeleton of 1,3-dimethylcyclobutane which could be generated on the sole basis of the geometries “2 × −CH$_2$”, “2 × −CH−”, and “2 × >C−” representing the hybridization states “2 × =CH$_2$”, “2 × =CH−”, and “2 × >C=”.

[9] Based on HSQC information, H,C correlations are translated into connectivities between the heteroatoms themselves. Hydrogen atoms are not treated as separate atoms, because the definition of atom types implies how many hydrogen substituents a heteroatom bears.

[10] COCON considers ^{13}C-NMR chemical shifts by exclusion of certain bonds between heteroatoms and carbon atoms in the following cases: $\delta_C < 150$: The carbon atom must not be connected to sp^2-hybridized oxygen or sulfur atoms. $\delta_C < 130$: If the carbon atom is sp^2-hybridized, it must not be connected to an oxygen atom. $\delta_C < 105$: If the carbon atom is sp^2-hybridized, it must not be connected to a nitrogen atom. $\delta_C < 45$: The carbon atom must not be connected to an oxygen atom. $\delta_C > 35$: If the carbon atom belongs to a methyl group, it must not be connected to another carbon atom.

[11] COCON contains an independent module which selects possible hybridization states on the basis of the following rules: (a) The total numbers of single-, double-, and triple-bond valencies represented by a hybridization-state combination must be even. (b) ^{13}C-NMR chemical-shift criteria (differing from those applied by COCON): $\delta_C < 180$: The carbon atom must not be an allene carbon atom. $\delta_C > 125$: The carbon atom must not be sp^3-hybridized. $\delta_C > 115$: The carbon atom must not be sp-hybridized. $\delta_C < 75$: The carbon atom must not be sp^2-hybridized.

[12] Of course, a COSY correlation between the two protons might not be observed if the dihedral angle is close to 90°. Application of the non-1,1-ADEQUATE option implies that every carbon−carbon bond involving at least one proton-bearing carbon atom will be forbidden, if no 1,1-ADEQUATE correlation is observed. This may be critical if the chemical shifts of two carbon atoms involved in the double quantum coherence are identical. Then the approximation of a weakly coupled spin system ($\Delta\delta >> J$) does not hold anymore. As a result, the corresponding cross peak cannot be observed (as it is already known from the INADEQUATE experiment). This case should be very rare, because the chemical-shift dispersion in the ^{13}C dimension is quite large.

[13] The size of the total free sphere be the sum of the sizes of the first-sphere and the second-sphere segments of an atom.

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