Assignment of the ^1H and ^{13}C Nuclear Magnetic Resonance Spectra of Norethisterone using Two-dimensional Nuclear Magnetic Resonance Spectroscopy

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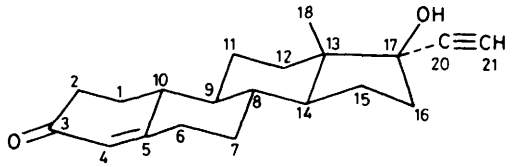
The information obtained from 2D *J*-resolved and 2D spin-echo-correlated spectroscopy at 500 MHz allows the complete assignment of the ^1H n.m.r. spectrum of norethisterone. In turn, the ^1H n.m.r. assignment readily leads to the assignment of the ^{13}C n.m.r. spectrum of this compound by means of a two-dimensional hetero-shift correlation experiment.

In our study of the photodecomposition of norethisterone (**1**), a frequently used progestogenic steroid of the oral contraceptive pill, a method was needed that enabled the identification of the 19-norsteroidal photo-products. Recently, the use of two-dimensional *J*-resolved n.m.r. spectroscopy (2D-*J*) in combination with nuclear Overhauser enhancement difference spectroscopy (NOEDS) has been advocated [1-3] for analysing the steroid skeleton. Moreover, an n.m.r. assignment strategy based on these techniques was proposed.[1]

However, with the advent of two-dimensional n.m.r. techniques a more straightforward and less time-consuming assignment–analysis of the ^1H n.m.r. spectrum of steroid-type molecules seemed [4] possible. In the present communication we report the complete analysis of the ^1H n.m.r. spectrum of (**1**) on the basis of a 2D *J*-resolved [5,6] and a 2D spin echo *J*-correlated (SECSY) experiment.[7,8] It will be shown that the ^1H resonance assignment can be used to assign the ^{13}C n.m.r. spectrum by means of a 2D ^1H–^{13}C hetero-shift correlation experiment.[9,10]

Our choice of (**1**), a 19-norsteroid, as a model compound for this study was dictated by the following considerations: in comparison with the steroid molecules hitherto analysed by ^1H n.m.r., the absence of the 10-methyl group and some other substituents infers a much more complicated network of coupled spins (Figure 1, all but 4-H resonating in the crowded high-field region of the spectrum); (**1**) is the 'parent' compound in our photodecomposition studies. Taken together, we feel that (**1**) represents a good test case for our assignment procedure.



(**1**)

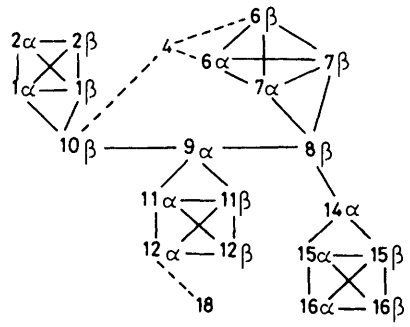


Figure 1. Diagram showing the coupling constant network for the protons in (**1**); long-range couplings are indicated by dashed lines

Experimental

Norethisterone (**1**) was purchased from Sigma and used without further purification. Samples were prepared by dissolving (**1**) (*ca.* 15 mg) in CD_3OD (1 ml).

^1H N.m.r. spectra were recorded on a Bruker WM-500 n.m.r. spectrometer interfaced to an ASPECT-2000 computer and a real-time pulser board. Chemical shifts (δ) were measured relative to the residual methanol peak and converted to the standard tetramethylsilane scale by adding 3.38 p.p.m.

The 2D *J*-resolved spectrum of (**1**) was recorded using the pulse sequence $[RD - 90° - t_1/2 - 180° - t_1/2 - t_2]_n$ where t_1 and t_2 denote the evolution and observation period, respectively; $t_1/2$ was varied between 8 and 512 ms in steps of 8 ms; for each value of t_1 32 free induction decays (8k datapoints, sweepwidth 4 000 Hz, 1.024 s acquisition time) were accumulated. A relaxation delay (RD) of 2.1 s was applied. After apodisation (sine bell window in both dimensions), the free induction decays were zero-filled to 16k (in t_2) and 128 (in t_1) datapoints and Fourier transformed.

In the SECSY experiment the standard pulse scheme of Nagayama *et al.*[7] was used: $[RD - 90° - t_1/2 - 180° - t_1/2 - t_2]_n$. For each value of t_1 16 free induction decays (4k datapoints, sweepwidth 5 000 Hz, acquisition time 0.41 s) were recorded; a relaxation delay (RD) of 2 s was inserted between transits. The values of $t_1/2$ was varied between 0.2 and 51.2 ms in steps of 0.2 ms. Free induction decays were multiplied by a sine bell window function in both dimensions and zero-filled to 1k datapoints in the t_1 dimension prior to Fourier transformation.

^{13}C N.m.r. spectra were recorded on a Bruker WM-200WB spectrometer operating at 50.3 MHz also equipped with an ASPECT-2000 computer and a real-time pulser board. Chemical shifts were measured relative to the central peak of the methanol multiplet and converted to the tetramethylsilane scale (δ_{CH_3OH} 49.3 p.p.m.).

The 2D ^1H–^{13}C hetero-shift correlation spectrum was recorded using the pulse sequence and phase cycling proposed by Bax;[10] 64 spectra were recorded (1 376 scans, 2k data points, sweepwidth 12 195 Hz, acquisition time 84 ms) with a $t_1/2$ increment of 300 μs. A relaxation delay of 0.8 s was allowed for between transits. Before Fourier transformation the time-domain spectra were multiplied by a phase-shifted sine bell

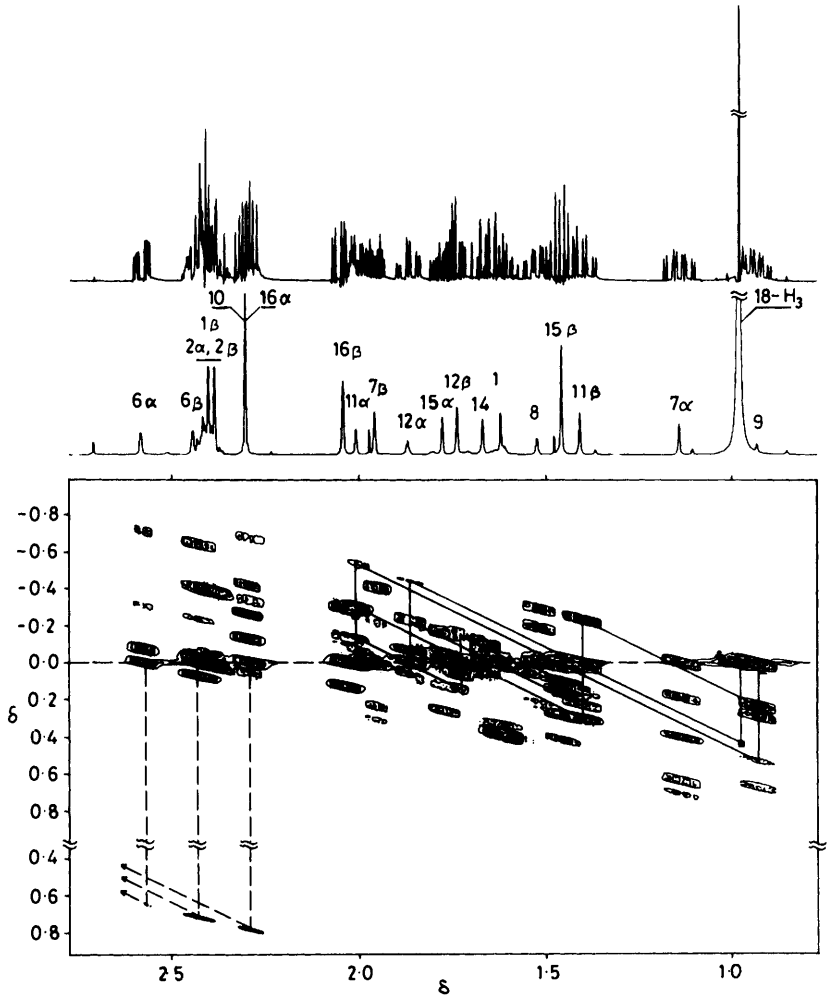


Figure 2. High-field part of the 500 MHz SECSY spectrum of (1) presented as an absolute value contour plot. For reference purposes the 1D 500 MHz n.m.r. spectrum (top trace) and the projection of the 500 MHz 2D *J*-resolved spectrum of (1) are plotted along the horizontal (f_2) axis. Solid lines interconnect the cross-peaks used for assigning the 18-, 12α-, 12β-, 11α-, 11β-, and 9-H (see text). Dashed lines indicate the interconnections (*via* long-range couplings) between the vinylic 4-H (not shown) and 6α-, 6β-, and 10-H

$\{\sin[\pi(t + t_0)/t_s]$, where t_s is the experimental acquisition time and $t_0/t_s = 1/3\}$ and zero-filled to 4k (in t_2) and 128 (in t_1) datapoints, respectively.

Results and Discussion

The top trace in Figure 2 shows the high-field region (0.9—2.5 p.p.m.) of the normal one-dimensional 500 MHz ^{1}H n.m.r. spectrum of (1). It is seen that even at 500 MHz this spectral region is still very crowded due to the many overlapping signals of the 23 protons resonating in this part of the spectrum.

A considerable simplification is achieved in the 2D *J*-resolved spectrum. The projection of the 2D-*J* spectrum on the f_2 axis (δ) results in a 'broad-band proton-decoupled' ^{1}H n.m.r. spectrum (Figure 2, middle) in which each proton is represented by a singlet at its intrinsic chemical shift position. Virtually all signals are well resolved in this projection, thus allowing an accurate determination of their chemical shifts.

Cross-sections parallel to the f_1-axis can be extracted from the 2D *J*-resolved spectrum for most of the protons. In the latter cross-sections the protons show up as first order multiplets from which the ^{1}H–^{1}H coupling constants for the proton at issue can be obtained. However, not all coupling constants could be determined because some protons (16α- and 10-H; 2α-, 2β-, and 1β-H) resonate at almost the same frequency, so that for these protons only intermingled cross-sections could be obtained. The chemical shift and coupling constant data determined for (1) are summarized in Tables 1 and 2; the chemical shift data are considered accurate to 0.001 p.p.m., the coupling constant data to *ca.* 0.4 Hz.

Although the 2D *J*-resolved n.m.r. experiment greatly reduced the complexity in comparison with its one-dimensional counterpart, it yields but little information which can be used for direct assignment purposes. Therefore, a SECSY spectrum of (1) (Figure 2) was recorded to establish the *J* connectivities between individual proton signals. The complicated pattern of cross-peaks manifested in the SECSY spectrum (Figure 2) is what one may expect on the basis of the molecular structure of (1) from which a complex and extended network of coupled spins (Figure 1) may be inferred. At this point the assignment problem boils down to an interpretation of the SECSY cross-peaks (Figure 2) in keeping with the expected network of coupled spins (Figure 1).

In the present case only two obvious assignments are available as starting points for the analysis of the SECSY spectrum: (a) the vinylic 4-H resonating at δ 5.876 (not shown) which displays allylic couplings to 6α-H (< 0.4 Hz), 6β-H (*ca.* 2.1 Hz), and 10-H

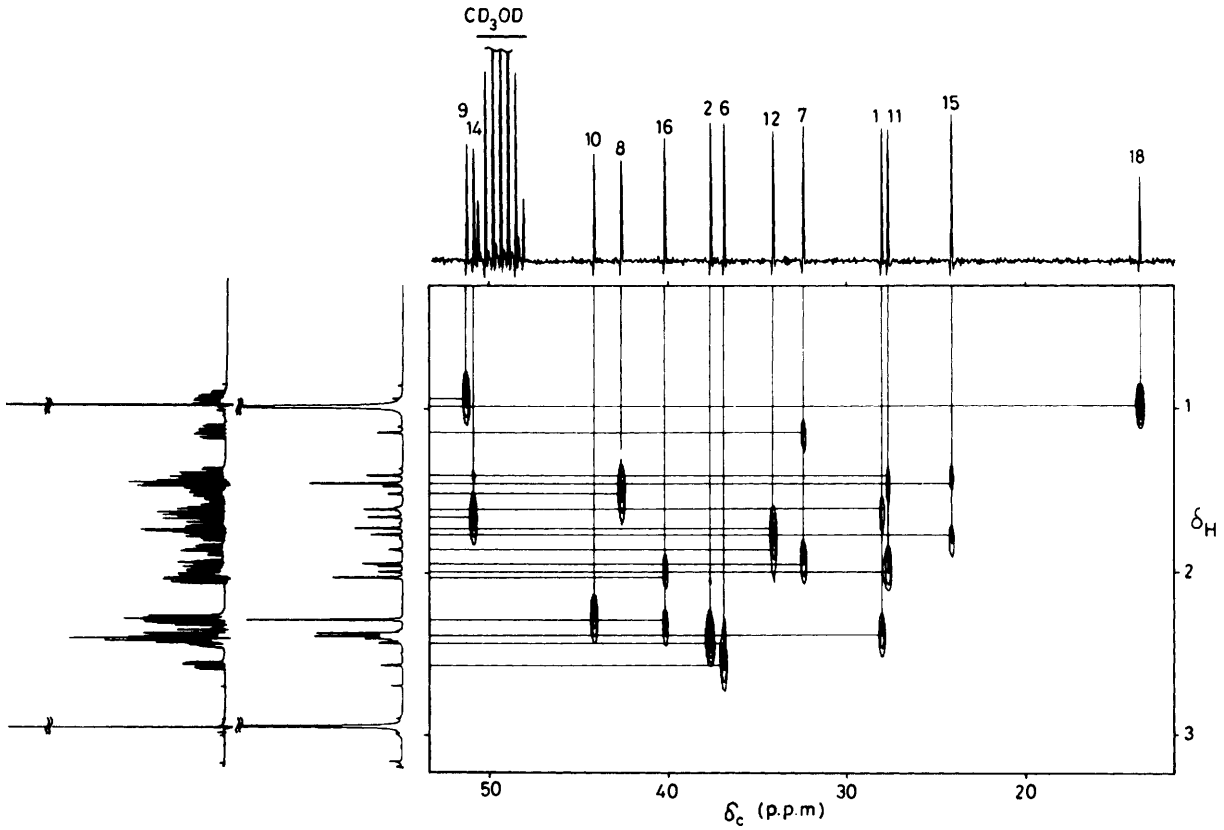


Figure 3. Part of the 200/50.3 MHz ^1H–^{13}C shift-correlated 2D-n.m.r. spectrum of (**1**) in the form of an absolute value contour plot. For reference purposes a 50.3 MHz ^{13}C-{^1H} n.m.r. spectrum is plotted along the horizontal (f_2) axis and a 500 MHz ^1H n.m.r. spectrum together with the projection of the 500 MHz 2D *J*-resolved n.m.r. spectrum (*cf.* Figure 2) are plotted along the vertical (f_1) axis. The cross-peaks in the contour plot indicate coherence transfer between a specific carbon atom and its directly bound hydrogen *via* the $^1J_{CH}$ scalar coupling

Table 1. Chemical shift data for (**1**)

Carbon	δ (p.p.m.)	Proton	δ
1	28.0	1α	1.614
		1β	*ca.* 2.40[b]
2	37.6	2α	*ca.* 2.40[b]
		2β	*ca.* 2.40[b]
3	203.1		
4	125.1	4	5.876
5	170.9		
6	36.8	6α	2.576
		6β	2.437
7	32.4	7α	1.136
		7β	1.951
8	42.6	8	1.517
9	51.2	9	0.929
10	44.1	10	2.296
11	27.7	11α	2.000
		11β	1.403
12	34.1	12α	1.862
		12β	1.730
13	N.m.[a]		
14	50.8	14	1.662
15	24.1	15α	1.770
		15β	1.453
16	40.1	16α	2.296
		16β	2.036
17	80.5		
18	13.5	18	0.977
20	89.0		
21	75.1	21	2.953

[a] Not measured (signal hidden by the solvent signal). [b] Not resolved.

(*ca.* 2.1 Hz) (*cf.* Figure 2); (b) the 13-methyl protons resonating at δ 0.977. It is well established [2] that the latter methyl group is long-range-coupled to 12α-H. As indicated in Figure 2, the corresponding cross-peaks in the SECSY spectrum therefore unambiguously assign the resonance at δ 1.862 to 12α-H. In accordance with the coupling constant network shown in Figure 1, the 12α-H resonance displays three other cross-peaks in the SECSY spectrum, in other words *J*-couplings to the resonances at δ 1.403, 1.730, and 2.000 are manifest. The resonance at δ 1.730 has only three cross-peaks (indicating couplings to three protons), the resonances at δ 1.403 and 2.000 each have four cross-peaks (indicating couplings to four other protons). From the expected *J*-coupling network (Figure 1) it is evident that the resonance at δ 1.730 has to be assigned to the 12β-H resonance, the resonances at δ 1.403 and 2.000 must arise from 11α-and 11β-H. Discrimination between the latter two protons can be made on the basis of the coupling constants extracted from the 2D *J*-resolved spectrum: the resonance at δ 1.403 displays three large couplings (*ca.* 13.3 Hz each) and one small coupling (4.2 Hz). Obviously, one of the large coupling constants is due to the geminal 11α-H–11β-H coupling, the remaining large coupling constants must arise from couplings between axial-axial orientated vicinal protons. This immediately assigns the resonance at δ 1.403 to the (axial) 11β-H. In accord with this assignment the resonance at δ 2.000 displays only one large coupling ($J_{11_\alpha,11_\beta}$ 13.3 Hz) and three relatively small couplings (2.8—4.2 Hz, equatorial-axial and equatorial-equatorial type ^3J), indicating that this proton occupies the equatorial position at C-11 (*i.e.* 11α-H). 11α- and 11β-H each have one cross-peak left which has not yet been used for assignment purposes. These cross-peaks connect both protons

Table 2. Geminal, vicinal, and long-range coupling constants in (**1**) (in Hz)

Proton pair	^2J/Hz	^3J/Hz ax-ax	^3J/Hz ax-eq	^3J/Hz eq-eq	Long range (Hz)
4, 6α					<0.4
4, 6β					2.1
4, 10					2.1
6α, 6β	−14.4				
6α, 7α			4.1		
6α, 7β				2.3	
6β, 7α		14.0			
6β, 7β			5.0		
7α, 7β	−12.5				
7α, 8β		11.8			
7β, 8β			3.5		
8β, 9α		9.9			
8β, 14α		12.3			
9α, 10β		10.2			
9α, 11α			4.2		
9α, 11β		13.2			
11α, 11β	−13.3				
11α, 12α			4.2		
11α, 12β				2.8	
11β, 12α		13.2			
11β, 12β			4.2		
12α, 12β	−13.2				
12α, 18					1.2
14α, 15α			7.4		
14α, 15β			10.7		
15α, 15β	−12.2				
15α, 16α			9.8		
15α, 16β			3.9		
15β, 16α			5.8		
15β, 16β			12.1		
16α, 16β	−13.6				

Individual coupling constants for 1α-, 1β-, 2α-, and 2β-H could not be determined due to the near isochronicity of the 1β-, 2α-, and 2β-H. The stereochemical orientation of the 1α-H was established on the basis of the total width of this resonance (indicating a sum of *ca.* 42 Hz for the coupling constants, which correspond to two axial-axial, one geminal, and one axial-equatorial coupling constant).

to the same resonance at δ 0.929, thereby identifying the latter resonance as arising from 9-H.

Pursuing this line of reasoning, all the remaining resonances in the spectrum of (**1**) can be assigned; the results are summarized in Table 1. However, we note that at some points in the analysis bifurcations may occur in which case the investigator either needs extra information (*e.g.* by starting from another 'obvious' assignment) or has to resort to deductive reasoning (*e.g.* by making an arbitrary assignment and pursuing the analysis until an obvious incongruity is encountered in which case the arbitrary assignment is changed). With respect to the latter method it should be stressed that the complexity of the network of coupled spins encountered in steroid molecules allows for so many cross-checks (on the basis of the SECSY connectivities as well as the coupling constant data obtained from the 2D *J*-resolved experiment) that an unambiguous assignment can be reached.

A detailed discussion of the experimental coupling constants and chemical shifts in terms of the molecular geometry of (**1**) is deemed beyond the scope of the present paper; however, it is noted that on first sight almost all experimental vicinal coupling constants have values which are close to the values one calculates on basis of a rough model of (**1**) using a generalized [11] Karplus equation. The remarkable exceptions are formed by the axial-axial couplings involving 9-H which are either smaller (experimental: $J_{8,9}$ 9.9, $J_{9,10}$ 10.2 Hz; calculated: *ca.* 11.9 Hz) or larger (experimental: $J_{9,11\beta}$ 13.2 Hz; calculated: *ca.* 12.3 Hz) than expected. For the time being no clear-cut explanation for this phenomenon can be offered; possibly subtle effects originating from the molecular geometry of (**1**) (such as atypical valency angles about C-9) are responsible for this aberrant behaviour.

Once the proton resonances are assigned, most of the C-13 resonances of (**1**) can be assigned in a simple and straightforward way on the basis of a 2D ^1H–^{13}C hetero-shift correlation experiment.[9,10] This is shown in Figure 3 where a part of the ^1H–^{13}C shift correlation spectrum of (**1**) is presented. The cross-peaks in this spectrum reveal the *J*-connectivities between the carbons and their directly bonded protons thus allowing the unequivocal assignment of the greater part of the ^{13}C resonances of (**1**) in a single experiment.

As the heteronuclear shift correlation experiment in Figure 3 was optimized to detect $^1J_{CH}$ of *ca.* 140 Hz, only those carbons directly bonded to one or more hydrogen atoms can be detected in this way. The remaining quaternary carbons (*i.e.* C-3, -5, -13, -17, and -20), however, resonate in very typical parts of the 1D ^{13}C n.m.r. spectrum and can therefore be easily assigned on the basis of their distinctive chemical shifts. The ^{13}C chemical shift data for (**1**) are summarized in Table 1; they show a gratifying correspondence with ^{13}C data reported for closely related steroids.[12]

The ^1H–^{13}C hetero-shift correlation experiment is in full accord with our analysis of the ^1H n.m.r. spectrum of (**1**), thereby lending further support to the correctness of this analysis. For example, the ^1H resonances assigned to 11α- and 11β-H both show cross-peaks to the same carbon resonance (C-11) thus endorsing their geminal relationship.

In the present paper we have used the hetero-shift correlation experiment to assign the ^{13}C spectrum of (**1**). It goes without saying that in those cases where specific carbon resonances are more readily assigned than the proton resonances, a ^{13}C–^1H shift correlation experiment can be employed to assign certain proton resonances, which in turn may be used as a 'starting point' in the analysis of *e.g.* the SECSY spectrum.

Conclusions.—Analysis of the complex n.m.r. spectra of steroid molecules by means of 2D-*J* and SECSY instead of NOEDS obviously has some advantages. The acquisition of the data will take a shorter period (the SECSY spectrum was recorded in less than 3 h) and is straightforward, requiring only a single instrument setting. Moreover, disturbances arising from selective transfer phenomena [13] or from 'spill-over' of irradiation power to neighbouring resonances, which are commonly encountered nuisances in NOEDS spectra, are non-existent in the SECSY experiment. On the other hand it should be pointed out that NOEDS provides a different type of information than 2D-correlated spectroscopy since the former technique establishes short internuclear distances between protons which are not necessarily scalar-coupled to each other. As such, NOEDS and SECSY yield complementary information as can be illustrated by considering for example the case of a 'normal' steroid with a 10-methyl group, where SECSY is, *a priori*, not capable of discriminating between the 1- and 2-H (NOEDS can; see *e.g.* refs. 1—3). But then, in the reverse case, *viz.* a 19-norsteroid such as (**1**), NOEDS is only of limited help in making the assignment in *e.g.* the A-ring of the steroid molecule due to the absence of a 10-methyl 'reporter' group, whereas the present paper shows that for (**1**) a SECSY experiment provides all the information needed for a complete assignment.

It is noted that the information obtained by either NOEDS or SECSY overlap to a great extent. Seen in the light of the faster data accumulation we therefore propose to use the 2D *J*-

resolved–SECSY combination as a first approach in solving the assignment problems encountered in steroid molecules. In those cases where this combination does not lead to a complete assignment, the obtained n.m.r. data can be complemented by a few selected NOEDS experiments.

When feasible, two-dimensional ^{13}C–^{1}H hetero-shift correlation experiments provide at least a thorough check on the attained ^{1}H n.m.r. analysis and may in some cases even be of great assistance in reaching a complete ^{1}H n.m.r. assignment.

As the photoreactive part of (**1**) is formed by the conjugated ketone function, photochemically induced changes are only expected to occur in the A-ring and B-ring of (**1**). We therefore expect that the analysis of the photoproducts of (**1**) will be facilitated by the present assignment procedure.

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