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Investigating the Sensitivity Limits of ^{13}C-Detected ^{1}H–^{13}C Chemical Shift Correlation Sequences with Modern Microprobe and Microtube Technology

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The sensitivity limits for ^{13}C-detected ^{13}C–^{1}H shift correlation sequences were evaluated using a microprobe in combination with 3 mm microtubes fitted with susceptibility plugs which allow for further restriction of the sample volume. For an absolute value BIRD-decoupled HETCOR spectrum acquired in 4–5 h, the sensitivity limit for compounds with non-equivalent CH_2 groups in *ca.* 3 μmol of sample, with significantly lower limits for compounds with only CH and/or CH_3 groups. For an absolute value *n*-bond spectrum acquired with the FLOCK sequence in 16 h, a similar amount of sample is barely adequate to observe cross peaks from CH_3 singlets while a spectrum showing a large number of *n*-bond peaks to CH, CH_2 and CH_3 protons requires about 15 μmol of sample. Further sensitivity gains could be realized by using phase-sensitive versions of the two sequences. While these experiments still fall considerably short of the corresponding limits for HMQC and HMBC using a similar ^{1}H-optimized microprobe, the ^{13}C-detected experiments can give significantly better ^{13}C resolution, hence they may still be advantageous for organic structure elucidation in cases where ^{13}C resolution is critical. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

^{13}C-detected correlation sequences for determining connectivities between directly bonded ^{13}C–^{1}H pairs[1] or between indirectly bonded ^{13}C–^{1}H pairs,[2,3] while previously widely used,[4] have now largely been supplanted by the significantly more sensitive ^{1}H-detected sequences such as HSQC,[5] HMQC[6] and HMBC.[7] Using modern microprobe technology, it is possible with the latter sequences to carry out organic structure elucidation with as little as 0.25 μmol of sample (e.g 100 μg for a compound of molecular weight 400).[8] The only major problem with the ^{1}H-detected sequences is the difficulty in obtaining adequate ^{13}C resolution. This is mainly due to the large number of time increments necessary to sample the wide ^{13}C spectral window. In addition, the intrinsic f_1 linewidth for HMQC and HMBC sequences is determined by the width of the ^{1}H multiplet structures since this multiplet structure appears along both f_1 and f_2 in multiple quantum sequences.[9] By contrast, the limiting f_1 resolution for the single quantum HSQC sequence is determined by the intrinsic ^{13}C linewidth,[9,10] although in practice it is difficult to reach this limit,[10] even with linear prediction.[11] Consequently, it may still be advantageous to use the ^{13}C-detected sequences in cases where excellent ^{13}C resolution is critical and the sample is not limited. For example, in a recent study of triterpenes, the ability to obtain one-bond and *n*-bond correlation spectra with 0.01 ppm resolution along both axes was critical for both structural and spectral assignments.[12] Finally, if one is working with samples of unknown compounds in the μmol range, it is usually essential to obtain a normal and a DEPT-edited spectrum, because of both the limited ^{13}C resolution of HMBC spectra and the fact that not all non-protonated carbons may show up clearly in an HMBC spectrum. ^{13}C spectra for such small samples are best determined using a ^{13}C-optimized microprobe.[8] Consequently, the possibility of obtaining ^{13}C-detected correlation spectra with this kind of microprobe is also worth considering.

These considerations led us to assess the sensitivity limitations of ^{13}C-detected ^{13}C–^{1}H correlation sequences, using modern microprobe and microtube technology. The results of this investigation are reported below.

RESULTS AND DISCUSSION

Our primary goal was to determine the minimum amounts of sample necessary to obtain a satisfactory ^{13}C-detected one-bond or *n*-bond ($n = 2$ or 3) corre-

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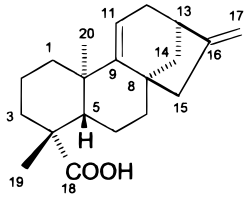
lation spectrum in a 16 h experiment, i.e. an overnight run. By extrapolation, about half this amount should be satisfactory for a weekend-long experiment of *ca.* 64 h, since the latter will allow the acquisition of four times as many transients per time interval, doubling the sensitivity. These times were chosen as representing, respectively, the usual maximum time available for a single experiment and the maximum time which might be devoted to a critical sample.

One-bond spectra were run using the BIRD-decoupled version of HETCOR[13] and *n*-bond spectra were obtained with FLOCK.[14] In each case, spectra were run in the absolute value mode, since this is the most commonly used version of each sequence, but the probable advantages of alternative sequences are also discussed.

Initial experiments focused on the minimum sample volume necessary to obtain ^{1}H spectra with satisfactory resolution. This is particularly significant for *n*-bond correlation experiments since poor resolution can lead to significant sensitivity loss during the relatively long fixed delay (0.064 s in this case) before polarization transfer from ^{1}H to ^{13}C. Although it is suggested that 65 μl is the minimum usable volume,[15] we found that this led to significant degradation of resolution, i.e. 1.25–1.5 Hz linewidth at half-height for the tetramethylsilane peak. The presumably reflects a small susceptibility mismatch between the sample solution and the susceptibility plugs of the 3 mm Shigemi tubes. The latter are nominally matched to the susceptibility of pure $CDCl_3$ but not necessarily precisely matched to $CDCl_3$ solutions. However, a small increase in volume to 70–75 μl improved the resolution of the Si $(CH_3)_4$ peak to 0.8–0.9 Hz, which was judged satisfactory for obtaining two-dimensional spectra.



We chose kauradienoic acid (**1**) as a test molecule since it is of intermediate size (molecular weight = 300) and considerable spectral complexity.[16] The initial test sample consisted of 1.05 mg (3.5 μmol) of **1** dissolved in 75 μl of $CDCl_3$ containing 0.1% TMS as internal reference. Two 16 h HETCOR experiments were run, one using the full spectral window and the other focusing on the aliphatic region (see Experimental for full details). Both gave very similar results; the contour plot for the aliphatic spectrum is illustrated in Fig. 1 and representative ^{13}C cross-sectional spectra are shown in Fig. 2. The C-6 cross-sectional spectrum is the one showing the poorest signal-to-noise ratio and yet this is still adequate. The peak at the middle of the C-6 AB quartet is the ubiquitous BIRD-decoupling artifact which has been explained by Bain *et al.*[17]

A repeat experiment in which the total acquisition time was reduced to 5 h (176 transients per time increment instead of 576 with all other parameters identical) gave a spectrum with marginal signal-to-noise ratio for H-6$_\beta$ but than more adequate sensitivity for all

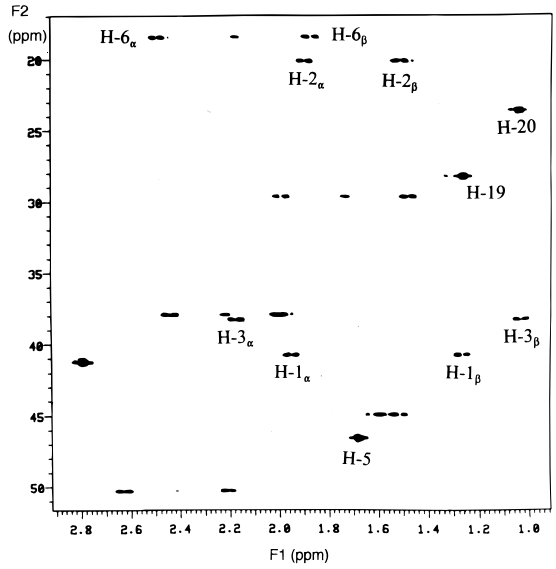


Figure 1. Contour plot for the aliphatic region of a 3.5 μmol sample of **1**, obtained with the BIRD-decoupled HETCOR sequence. Total acquisition time = 16 h. Protons included in cross-sections in other figures are labelled.

other cross peaks. In assessing these observations, one key point should be noted. The C-6 cross-sectional spectrum, and particularly H-6$_\beta$, consistently shows a signal-to-noise ratio that is half to two thirds of that for other CH_2 groups in **1** in both BIRD-decoupled HETCOR and fully proton-decoupled HETCOR spectra[18] and also in HMQC and HSQC spectra.[10] This may arise, at least in part, because H-6$_\beta$ is affected by virtual coupling.[10,19] However, regardless of the reasons, if one ignores this anomalous result, completely adequate absolute value HETCOR spectra of typical natural products should generally be obtainable in 4–5 h with 3–3.5 μmol of sample and with about half of this

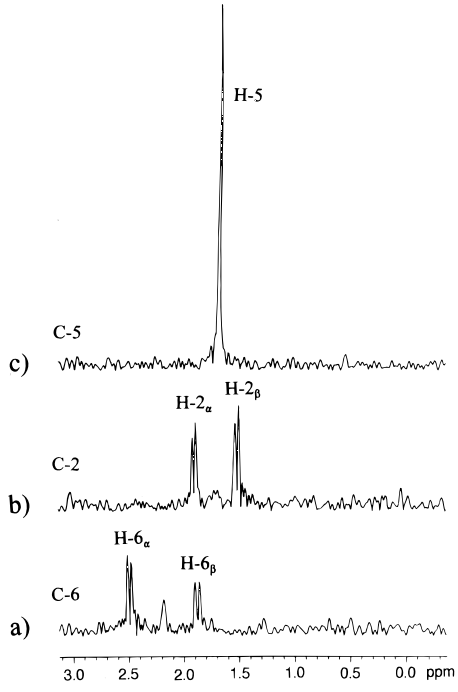


Figure 2. ^{13}C cross-sectional spectra from Fig. 1 for (a) C-6, (b) C-2 and (c) C-5.

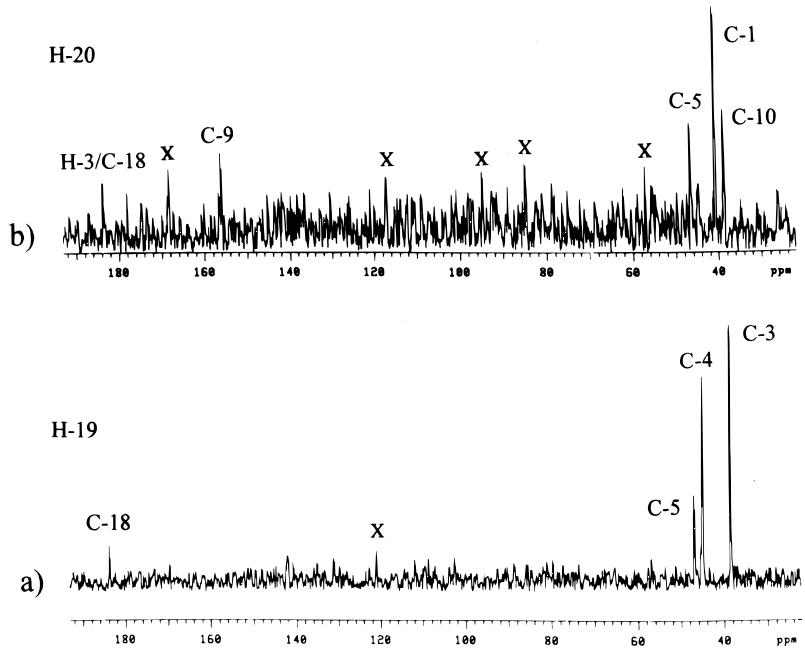


Figure 3. *n*-Bond methyl–^{1}H cross-sectional spectrum for a 3.5 μmol sample of **1**, obtained with the FLOCK sequence. Assigned carbons are listed beside each peak while the most intense noise spikes are marked X. (a) H-19, (b) H-20. The weak peak at δ 184 in (b) is an H-3$_\beta$–COOH cross peak, observed because H-3 has the same chemical shift as H-20 (see Fig. 1).

amount for an overnight run. Furthermore, even lower limits may be possible in special cases such as aromatic molecules and polysaccharides where most, if not all, of the protonated carbons have a single attached proton, since the intensities for CH peaks are typically more than double those of CH_2 peaks (e.g. see Fig. 2).

The same 3.5 μmol sample was used to obtain a 16 h *n*-bond FLOCK spectrum. As expected, the only clearly discernible cross peaks involved the two intense CH_3 singlets. Cross-sections through the two methyl ^{1}H signals are illustrated in Fig. 3. Both spectra show all four expected peaks (one two-bond and three three-bond peaks). However, particularly for H-20, there are spurious noise peaks which are close in intensity to the smaller of the real cross peaks. In this particular case, the noise peaks occur at frequencies at which no carbon signals are expected and thus cannot be mistaken for real peaks. Nevertheless, this might not always be true and consequently the spectra are only marginally acceptable. However, based on these results it should be possible to obtain a completely adequate FLOCK spectrum involving CH_3 singlets in an overnight run with no more than 5–6 μmol of sample and about half of this amount for a weekend run. For some types of natural products, e.g. diterpenes and triterpenes, *n*-bond CH_3 cross peaks are often sufficient, in conjunction with one-bond heteronuclear correlation spectra and COSY spectra, to complete structural and/or spectral assignments.[12,15,19,20]

However, at least 15 μmol of sample are required to obtain an overnight FLOCK spectrum containing a wide range of cross peaks involving CH, CH_2 and CH_3 groups. For example, Fig. 4 shows expansions of different regions of a FLOCK spectrum obtained with 5 mg (17 μmol) of **1** and Fig. 5 shows the corresponding cross-sectional spectra through H-19, H-20 and C-2. This spectrum clearly gives sufficient unambiguous

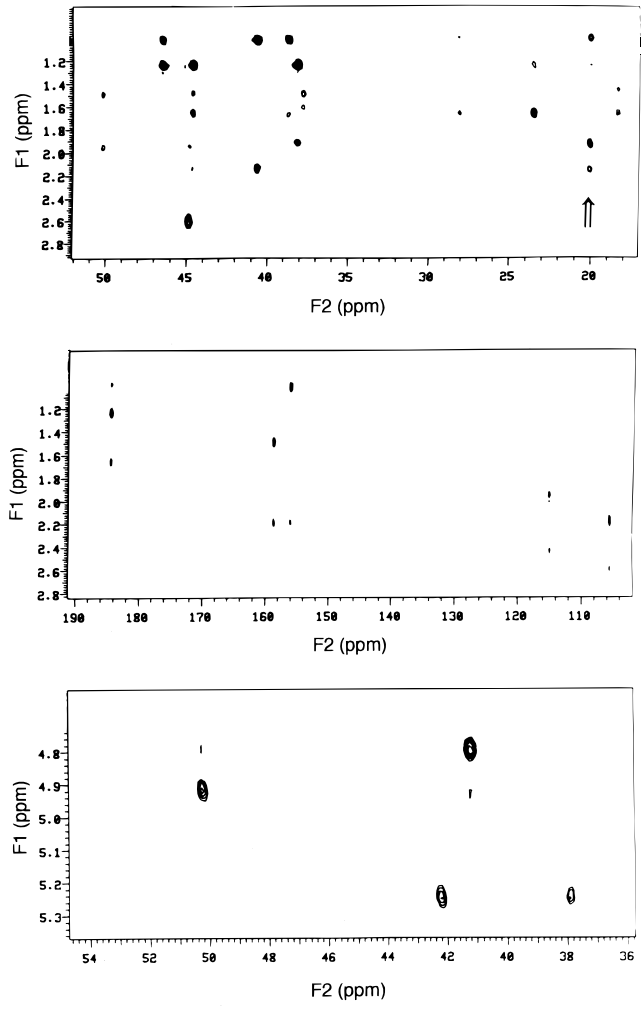


Figure 4. Expansions of the contour plot of a FLOCK spectrum obtained with a 16.7 μmol sample of **1**. The arrow marks the cross-section through C-2 illustrated in Fig. 5(c).

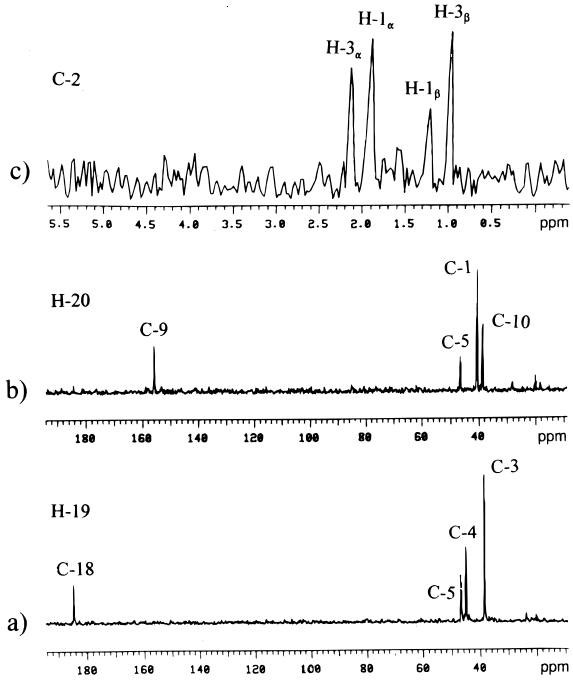


Figure 5. ^{1}H and ^{13}C cross-section spectra for the FLOCK spectrum in Fig. 4. (a) H-19 (δ 1.24); (b) H-20 (δ 1.02); (c) C-2 (δ 20.4). Weak peaks in (a) and (b) correspond to cross peaks with H-1_β and H-3_β, respectively, since the latter two ^{1}H signals overlap with H-19 and H-20, respectively (see Fig. 1). Note that the H-1_β–C-2 cross peak in (c) corresponds to the weakest cross peak observable in the contour plots illustrated in Fig. 4.

cross peaks to allow complete structural and spectral assignment with considerable redundancy.[16] A weekend spectrum should require about half of this amount of sample.

The main ^{13}C-detected alternative to FLOCK is the COLOC sequence, which involves a constant time between initial excitation and polarization transfer, leading to complete ^{1}H–^{1}H decoupling along f_1.[2] While this gives enhanced sensitivity for some cross peaks, relative to FLOCK, due to decoupling, others are considerably reduced in intensity, particularly when ${}^{n}J_{CH} \approx {}^{n}J_{HH}$ (where $n = 2$ or 3).[3] Hence there should be no significant overall advantage to COLOC over FLOCK. On the other hand, if one only needs to determine the n-bond cross peaks to a limited number of resolved ^{1}H multiplets, there will be significant advantages in carrying out a series of 1D selective INEPT experiments.[21] The ability to carry out the latter experiments is significantly aided by the capabilities of modern spectrometers of generating shaped pulses for selective excitation. It is difficult to generalize concerning the amount of sample required for an overnight run since this will depend upon the number of 1D spectra that are required. However, in favourable cases, the sample requirements should be as little as 1–3 μmol.

Comparing these results with the results given in Ref. 8, it appears that the sensitivity advantage of HMQC over HETCOR for CH_2 groups is *ca.* 4–5 and that for HMBC over FLOCK is *ca.* 15 for CH and CH_2 groups and even larger for CH_3 groups. These are smaller and larger, respectively, than the theoretical advantages of $(\gamma_H/\gamma_C)^{3/2} \approx 8$ for ^{1}H-detected experiments. However, the relative sensitivity of the different types of experiments is determined by a number of factors of which the relative intrinsic sensitivity of ^{1}H *vs.* ^{13}C detection is only one, albeit a particularly important one. For example, in comparing HETCOR with HMQC, a ^{1}H multiplet structure appears along both ^{1}H and ^{13}C axes with HMQC,[9,10] whereas with HETCOR, there is no multiplet structure along the ^{13}C axis and the BIRD-decoupled version of HETCOR eliminates all couplings except geminal couplings along the ^{1}H axis. These simplifications of cross peak structure combine to represent a significant sensitivity advantage for HETCOR relative to HMQC. However, HMQC is usually run in a phase-sensitive mode, which should result in an additional $2^{1/2}$ gain in sensitivity relative to absolute value HETCOR.[22] Furthermore, one usually processes absolute value HETCOR spectra with a weighting function (e.g. shifted sine-bell or shifted sine-bell squared) which partially suppresses the initial portions of the FID and interferogram in order to minimize the absolute value 'tails.' This results in some sensitivity loss relative to HMQC spectra, which are usually processed with a less extreme weighting function such as Gaussian multiplication or exponential line broadening. Similar arguments apply to the comparison of FLOCK (or COLOC) and HMBC but with one important additional consideration. HMBC detects couplings to a specific proton from a single ^{13}C nucleus whereas with FLOCK (and with COLOC), the intensity of a given ^{1}H–^{13}C cross peak (the active coupling) is affecting by the coupling of all other protons to the detected ^{13}C (passive couplings). The presence of passive coupling forces one to use a delay after polarization transfer which is considerably smaller than optimum for an isolated ^{1}H–^{13}C pair and significantly decreases the intensity of the cross peak.[23] It appears that this combination of factors results in a smaller than theoretical sensitivity difference between HETCOR and HMQC but a larger difference between FLOCK (or COLOC) and HMBC. The greater sensitivity advantage for CH_3 groups reflects the fact that they show an additional factor of 3 sensitivity gain in ^{1}H *vs.* ^{13}C detected experiments.

Finally, we have previously demonstrated that phase-sensitive HETCOR spectra show the expected $2^{1/2}$ increase in sensitive relative to absolute value spectra[24] and similar sensitivity gains have been observed for phase-sensitive FLOCK spectra.[14] Although this has not been confirmed for the more dilute solutions used in this investigation, it seems reasonable to assume that phase-sensitive spectra would provide similar sensitivity advantages when using microprobe/microtube technology. If so, then this would reduce sample requirements to *ca.* 70% of the values quoted above.

CONCLUSIONS

Our overall conclusion is that modern microprobe and microtube technology have significantly lowered the sample requirements for ^{13}C-detected 2D experiments. While these requirements are still higher than for the corresponding ^{1}H-detected sequences, the latter advantage may in some cases be compensated by the

improved resolution of the former sequences.[12] In the case of the pharmaceutical industry, the ability to detect and identify sub-micromolar levels of compounds may be critical, e.g. for identifying drug metabolites, degradation products and synthetic process impurities. However, much of natural product research focuses not only on the isolation and identification of new compounds but also on subjecting them to a variety of pharmacological screenings. This typically requires several μmol of sample, which makes it feasible to carry out ^{13}C-detected shift correlation spectra. Thus, we believe that a ^{13}C microprobe would be a useful acquisition for any natural products chemist, both to provide high-quality ^{13}C spectra but also to provide ^{13}C-detected ^{13}C–^{1}H shift correlation spectra in cases where excellent ^{13}C resolution is required.

EXPERIMENTAL

All spectra were recorded on a Varian UNITY-500 spectrometer equipped with a Nalorac MD-500 3 mm probe optimized for ^{13}C–$\{^{1}$H$\}$ experiments (^{13}C 90° pulse width = 7.6 μs, ^{1}H 90° decoupler pulse width = 6.0 μs). The samples were prepared by dissolving a weighed amount of kauradienoic acid in 70–75 μl of $CDCl_3$ and transferring the solution by microsyringe to a Shigemi 3 mm microtube fitted with susceptibility plugs matched closely to the magnetic susceptibility of $CDCl_3$. All spectra were obtained non-spinning. HETCOR spectra were recorded using the standard BIRD-decoupled HETCOR sequence[13] included in the Varian software package. FLOCK spectra were recorded using the improved phase cycle version of this sequence.[14]

The HETCOR spectrum of **1** illustrated in Fig. 1 was obtained using a ^{13}C (f_2) spectral window of 7450 Hz, a ^{1}H (f_1) spectral window of 1745 Hz, a relaxation delay of 0.6 s, 1024 data points with zero filling to 2048, 128 time increments and 576 transients per increment, with the BIRD pulse optimized for $^{1}J_{CH} = 125$ Hz. The f_1 data were linearly predicted to 512 points with zero filling to 1024 and data were processed in absolute value mode with $\pi/3$ shifted sine-bell weighting along both axes. A corresponding spectrum for the entire proton/protonated carbon region (not shown) was obtained with $f_1 = 3018$ Hz, $f_2 = 15\,750$ Hz and with all other parameters identical with those listed above. The FLOCK spectra illustrated in Figs 3–5 were obtained with $f_1 = 3018$ Hz, $f_2 = 25\,000$ with 2048 data points (zero filled to 4096), a relaxation delay of 0.6 s, 128 increments, 512 transients per increment, BIRD pulses optimized for $^{1}J_{CH} = 140$ Hz and fixed delays before and after polarization transfer of 0.064 and 0.032 s, respectively. Again, the f_1 interferograms were linearly predicted to 512 with shifted sine-bell weighting used for both axes.

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