dinated (Griffith) structure for the dioxygen ligand geometry on the basis of total energies. For the bent, end-on structure, low-energy off-axis displacement of the dioxygen ligand and a low barrier to rotation about the Fe–O axis possibly coupled to a large amplitude bending mode of the Fe–O–O bond lead to a conformational flexibility of the dioxygen ligand. Both total and molecular energy values calculated from ab initio and iterative extended Hückel methods fail to resolve the uncertainties in the electronic ground-state configuration. However, only a totally paired iron(II)–dioxygen configuration can consistently account for the large negative electric field gradient at the iron nucleus observed as quadrupole splitting in Mössbauer resonance. In addition, the temperature dependence of the quadrupole splitting may be accounted for by rotation about the iron–oxygen bond. Due to covalency upon binding of iron to the ligands, the calculated electron distribution around the iron differs substantially from a low-spin iron(II) configuration. Finally, a large net negative charge calculated for the dioxygen ligand indicates possible superoxide character even without a charge transfer iron(III)–superoxide configuration.

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Nybomycin. 9. Synthetic and Biosynthetic Incorporation of ^{15}N as a Means of Assigning the ^{13}C Nuclear Magnetic Resonance Spectrum of Nybomycin[1,2]

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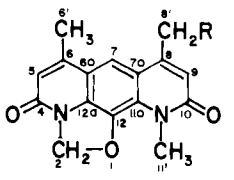
Abstract: The ^{13}C NMR spectrum of the antibiotic nybomycin (**1**) has been assigned unambiguously. Difficult central ring carbon assignments were made by incorporating ^{15}N biosynthetically and employing ^{13}C–^{15}N coupling constants. Other carbon assignments were made from off-resonance proton decoupling, model compound studies, and long range ^{13}C–^{1}H splitting patterns.

Our continuing biosynthetic investigation[2] of the antibiotic nybomycin (**1**) has required assignment of the ^{13}C NMR spectrum of nybomycin *n*-butyrate (**2**) to interpret the results



1: R=OH
2: R=$O_2C\overset{1''}{C}H_2\overset{2''}{C}H_2\overset{3''}{C}H_2\overset{4''}{C}H_3$

of ^{13}C precursor labeling experiments. Early studies of the biosynthesis dealt with the incorporation of labeled acetate and methionine into carbons 2, 4, 5, 6, 6′, 8, 8′, 9, 10, 11′, i.e., into the peripheral carbons of the antibiotic.[2b,c] For these studies it was sufficient to identify the peripheral ring carbons (in pairs) and less important to distinguish C-4 from C-10, C-5 from C-9, C-6 from C-8, since they were labeled symmetrically by acetate, or to assign the central ring carbons definitively, since they were unlabeled. However, later biosynthetic studies demonstrating incorporation of glucose and pyruvate into the central ring[2a] required complete, unambiguous assignment of C-6a, C-7, C-7a, C-11a, C-12, and C-12a and a more extensive investigation of the ^{13}C NMR spectrum of nybomycin was undertaken.

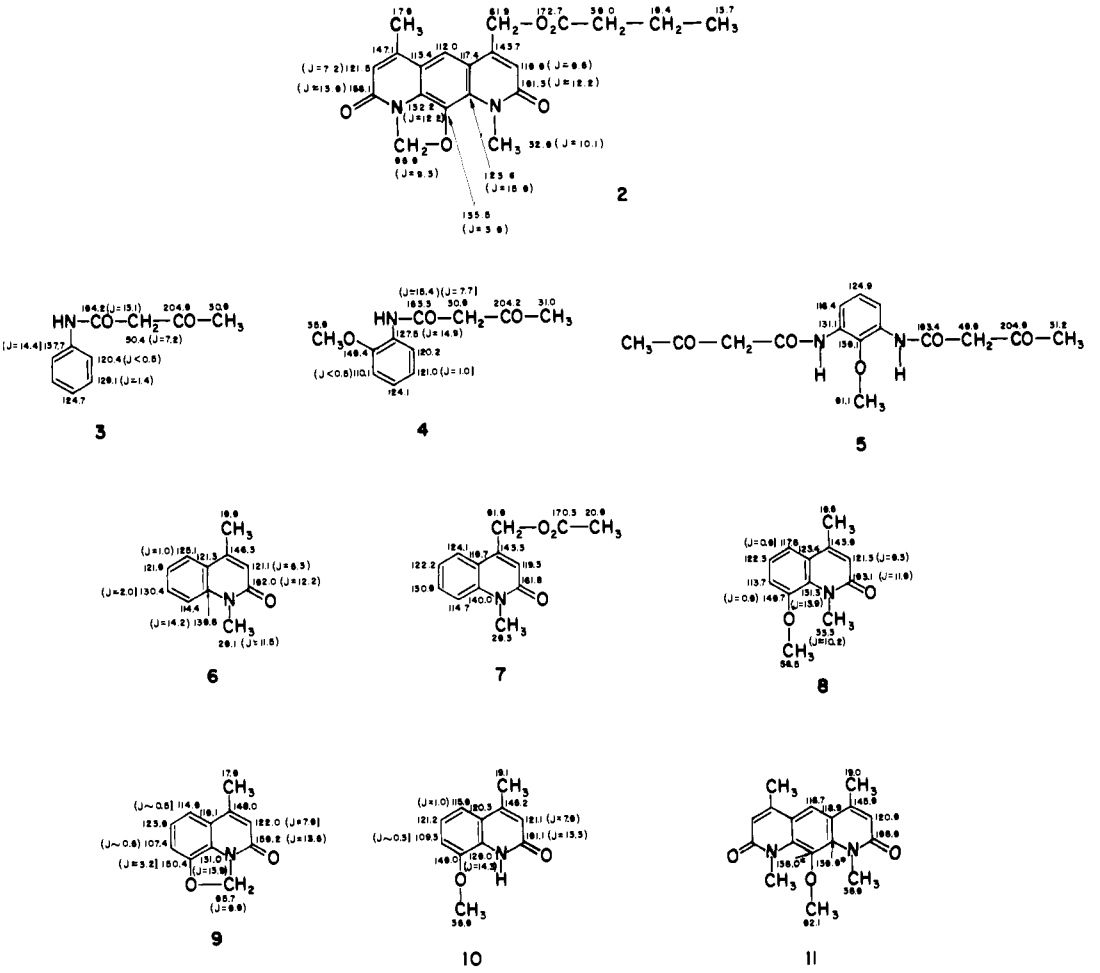


Figure 1. ^{13}C NMR resonances and ^{13}C–^{15}N coupling constants (J, in hertz) for nybomycin *n*-butyrate (**2**) and model compounds. Interchangeable assignments are indicated by asterisks. Several assignments for **2** are revised from previous assignments[2b,c] by interchanging C-5 and C-9, by interchanging C-6 and C-8, and by definitively assigning C-11a, C-12, and C-12a (see text).

Standard techniques involving examination of the off-resonance proton-decoupled and proton-coupled spectra of **2** and careful comparison of resonances with those of closely related model compounds, which we shall describe in the next section, permitted reliable assignment of all carbon atoms in **2**, except for three crucial resonances: C-11a, C-12, and C-12a (see numbering scheme). In order to identify these resonances we have biosynthetically incorporated ^{15}N into nybomycin in order to take advantage of the observable spin–spin coupling between ^{13}C and ^{15}N in compounds with a high level of ^{15}N. By comparing the ^{13}C–^{15}N coupling constants of biosynthetically ^{15}N-enriched nybomycin *n*-butyrate with those of closely related synthetically ^{15}N-labeled model compounds, we have completed the assignment of the nybomycin ^{13}C spectrum, including C-11a, C-12, and C-12a (see Figure 1). The present paper then describes this novel application of the new technique as well as the complete assignment of the ^{13}C NMR spectrum of nybomycin *n*-butyrate using ^{13}C–^{15}N coupling constants, model compounds, long range ^{13}C–^{1}H coupling patterns, and off-resonance proton decoupling.

Results and Discussion

Preliminary Studies. The poor solubility characteristics of nybomycin (**1**) require conversion to its soluble *n*-butyrate (**2**) for spectral determination. The proton-decoupled spectrum of nybomycin *n*-butyrate (Figure 2) shows the expected 20 singlet absorptions, while off-resonance proton decoupling establishes methyl, methylene, methine, and quaternary carbon resonances (Figure 2). The ^{13}C NMR absorptions of methyl *n*-butyrate[4] identify the four butyrate carbons of **2**, while the remaining two methyl absorptions are assigned as C-6′ (17.8 ppm) and C-11′ (32.6 ppm) by comparison to methyl absorptions of 1-methylnaphthalene[5] and 1,3-dimethyluracil.[5] The two methylene peaks at 61.9 and 85.9 ppm may be attributed to C-8′ and C-2, respectively, in view of the position of the methylene absorption in benzyl acetate[5] and the large, predictable deshielding effect of having both oxygen and nitrogen bound to C-2. Of the three methine carbons, downfield resonances 118.6 and 121.5 ppm are assigned to C-5 or C-9 by comparison to values for pyridone[6] itself, while the remaining absorption (112.0 ppm) is due to C-7. The absorptions of pyridone[6] also permit identification of the two pairs 158.1 and 161.3 ppm as C-4 or C-10 and 143.7 and 147.1 ppm as C-6 or C-8 (Figure 1).

Assignment of the remaining five quaternary centers (central ring) proves difficult due to the breakdown of the substituent additivity relationship for ortho-substituted aromatic systems.[7] Qualitatively, however, absorptions due to carbons attached to oxygen or nitrogen (C-11a, C-12, C-12a) should be downfield from those at C-6a and C-7a. At this stage model compounds more closely related to nybomycin are necessary to establish the remaining unassigned resonances, to assign specific resonances to carbons of the individual pyridone rings of **2**, and to verify the preliminary assignments based on simple model compounds.

Detailed examination of the ^{13}C NMR resonances of the substituted benzenes **3–5**, the quinolones **6–10**, and the diazaanthracenedione **11** permitted assignment of most of the

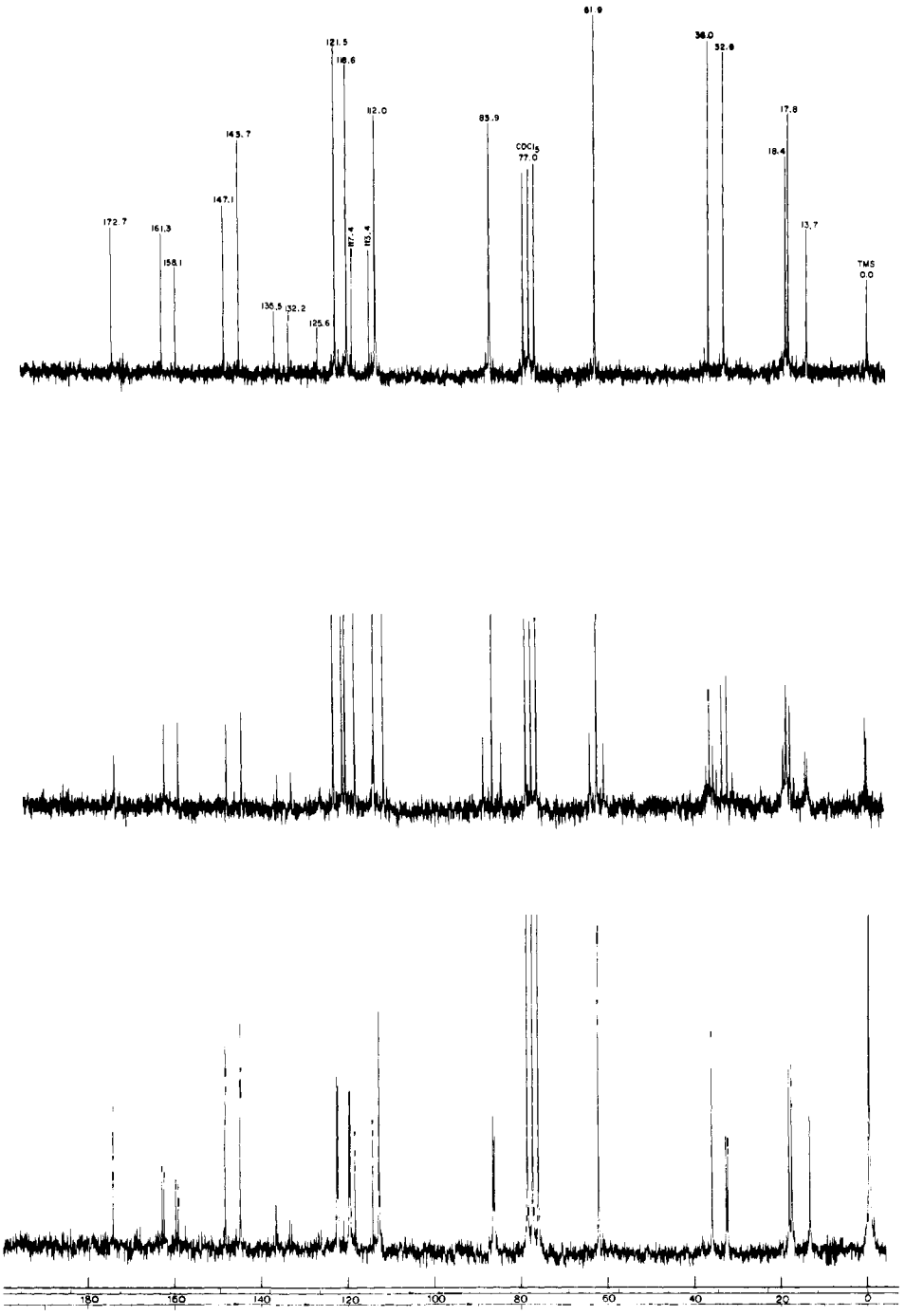


Figure 2. (Upper trace) proton decoupled and (middle trace) off-resonance decoupled ^{13}C FT NMR spectra of nybomycin *n*-butyrate; (lower trace) proton-decoupled ^{13}C NMR spectrum of [3,11-$^{15}N_2$]nybomycin *n*-butyrate (parts per million from Me_4Si).

resonances of **2**. Chemical shift assignments of these models were made in the following manner.

Proton-decoupled ^{13}C NMR spectra were determined for compounds **3–11** and off-resonance proton-decoupled spectra for all but **3** and **7** (Figure 1). Established resonances of acetanilide and ethyl acetoacetate[5] permit assignment of the carbons of acetoacetanilide (**3**) which, in turn, affords substituent additivity parameters for the acetoacetamido group (C-1, 9.0; *o*-, −8.3; *m*-, 0.4; and *p*-, −4.0). Ortho-substituted anisoles **4** and **5** may be assigned in accord with calculated values using additivity parameters for the methoxyl[8] and acetoacetamido functions. The ambiguity concerning the resonances of C-5 and C-6 in 2-methoxyacetoacetanilide (**4**) is readily clarified by a specific proton decoupling experiment, since irradiation of H-6, the most deshielded aromatic proton[9] in **4**, collapses to a sharp singlet the resonance centered at 120.2 ppm. Assigned resonances of **4** and **5** exhibit the expected deviations observed for other ortho-substituted and ortho-disubstituted anisoles.[10]

1,4-Dimethyl-2-quinolone (**6**) serves as a model for establishing resonances of **7–11**. Nonaromatic resonances of **6** are identified by analogy to pyridone,[6] 1-methylnaphthalene,[5] and 1,3-dimethyluracil.[5] Aromatic resonances can be correlated to those calculated for 2-acetoacetamidocinnamic acid from additivity relationships.[11,12] Ambiguous assignments for C-3, C-6, and C-8 in **6** are partially resolved by examination of the proton-coupled ^{13}C NMR spectrum. The absorption centered at 121.1 ppm exhibits coupling to both the olefinic proton (H-3, $^1J_{CH}$ = 162 Hz) and the methyl protons on C-4′ ($^3J_{CH}$ = 5.5 Hz) and, therefore, can be assigned to C-3. Resonances at

121.9 and 114.4 ppm, each appearing as a doublet of doublets due to one-bond ($^1J_{CH} \sim 165$ Hz) and three-bond ($^3J_{CH} \sim 7$ Hz) couplings, are attributed to C-6 and C-8. A second potential ambiguity, between C-5 and C-7, and C-6 and C-8 in **6**, may be resolved by observing the "fingerprint" couplings of the aromatic resonances in a modified continuous wave (CW) off-resonance decoupling experiment.[13] Application of this technique to **6** identifies resonances at 114.4 and 125.1 ppm as α and α' carbons and those at 121.9 and 130.4 ppm as β and β' carbons, thus providing reliable assignment of C-5, C-6, C-7, and C-8.

The absorptions of **7** can be established by observing resonance shifts (**6** to **7**) near C-4 of **6**. Substituent additivity calculations[12] on observed resonances of **6**, i.e., introduction of a methoxyl function at C-8, provide a basis for assigning the resonances of **8**, **9**, and **10**. Distinction between quaternary centers C-4 and C-8 in **8** is readily achieved by performing a specific proton decoupling study.[14] Irradiation of the C-methyl protons produces sharp singlets of enhanced intensity for the methyl carbon (C-4′) and the quaternary carbon attached to it (C-4, assigned as 145.8 ppm), while the resonance at C-8, assigned as 148.7 ppm, still exhibits broadening due to long range coupling to aromatic protons. This experiment also enables the resonances at 121.3 and 122.3 ppm to be distinguished, since irradiation of protons attached to C-4′ also results in a sharp doublet for C-3 (121.3 ppm), while the doublet centered at 122.3 ppm is transformed to a multiplet with considerable fine structure, indicative of a carbon coupled to a tightly proton-coupled system.[13a] The validity of this assignment is further confirmed by the observation of a doublet of quartets ($^1J_{CH} = 166$, $^3J_{CH} = 5.5$ Hz) centered at 121.3 ppm in the coupled spectrum of **8**. Analogous interchangeable assignments in **9** and **10** are resolved in an identical manner.

Another difficulty in substituent additivity calculations arises between C-5 and C-7 in **8** and C-7 and C-9 in **9**. Peak assignments can be made, however, on the basis of a large expected (upfield) deviation for the methine carbon ortho to the methoxyl function, in accord with the observations of Dhami and Stothers[10a] for a number of ortho-substituted anisoles.

The ^{13}C NMR spectrum of the symmetrical diazaanthracenedione **11** can be assigned readily with the aid of off-resonance decoupling and compound **8**, except that specific resonances cannot be assigned from these considerations to the three aromatic carbons bound to heteroatoms.

Resonances of the separate pyridone rings in nybomycin butyrate (**2**) are assigned by careful comparison to the above models. Carbonyl absorptions of **8** and **9** show that C-4 and C-10 in **2** absorb at 158.1 and 161.3 ppm, respectively. Assignment of C-6 and C-8 (147.1 and 143.7 ppm, respectively) in **2** is based upon magnitudes in shifts observed for **6** vs. **7** and **8** vs. **9**. Acetoxylation of **6** produces significant ($\geq$1 ppm) upfield shifts for C-3, C-4, C-5, and C-10 (of **6**), while formation of a fused oxazoline ring in **8** (i.e., to give **9**) produces upfield shifts of all carbons except for C-3, C-4, C-6, and C-8, which are shifted downfield slightly. The magnitudes of these shifts also permit differentiation between C-5 (121.5) and C-9 (118.6), and between C-6a (113.4) and C-7a (117.4 ppm) in nybomycin butyrate (**2**, Figure 1). The assignments of C-6 and C-8, and of C-5 and C-9 also are consistent with long range ^{13}C-^1H splitting patterns observed in the proton-coupled spectrum of **2** (C-6, dq, $^2J_{CH(6')} = 6.5$, $^3J_{CH(7)} = 4.0$ Hz; C-8, br q, $^2J_{CH(8')} = 4$, $^3J_{CH(7)} = 4$ Hz; C-5, dq, $^1J_{CH(5)} = 167$, $^3J_{CH(6')} = 6.5$ Hz; C-9, dt, $^1J_{CH(9)} = 169$, $^3J_{CH(8')} = 3.5$ Hz). All of the above long range splitting patterns and the magnitudes of the C-H coupling constants (J_{CH}) are in concert with observations of significant, albeit small, couplings in related olefinic and aromatic systems.[4,15]

Absorptions of nybomycin butyrate can actually be calculated from the structural variations observed in the above models; this may be performed in two different ways. Resonances of **11** can be adjusted for introduction of an ester function (**6** vs. **7**) and formation of an oxazoline ring (**8** vs. **9**). Alternatively, resonances of **9** can be adjusted for introduction of a second pyridone ring onto the structure (shifts calculated from **8** vs. **9**) with subsequent acetoxylation of the methyl function of the newly added ring (**6** vs. **7**). The two procedures afford calculated values[12] within 2 ppm of one another for all carbons and within about 1 ppm of the observed carbon resonances in **2** except for C-10, C-11′, and aromatic carbons C-11a, C-12, and C-12a. Calculated resonances for the latter three carbons are 129, 137 (138), and 136, respectively, and although the observed resonance at 125.6 probably can be attributed to C-11a, distinction between C-12 and C-12a (observed, 132.2 and 135.5) cannot be made on the basis of model studies. Since the identity of the aromatic carbons was critical to the success of the ^{13}C biosynthetic study, as noted at the outset, our attention was directed to exploring ^{15}N labeling as a method for defining unambiguously resonances at C-11a, C-12, and C-12a.

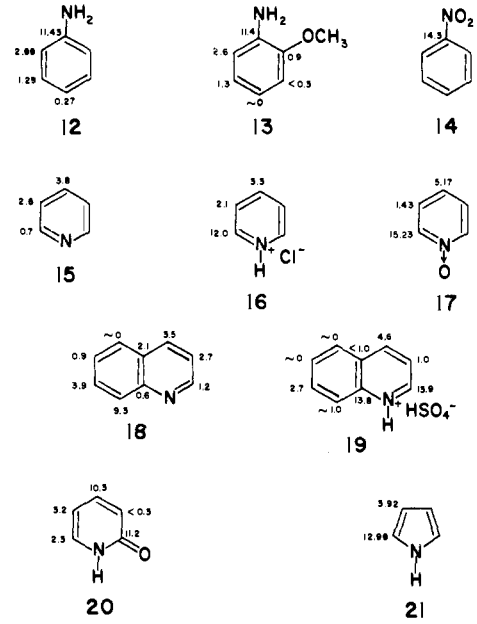


Figure 3. Selected ^{13}C-^{15}N coupling constants (in hertz) for aromatic and heteroaromatic compounds **12**–**21**.

Nitrogen-15 Labeling Studies. Identification of nitrogen-bonded aromatic carbons in **2**, using ^{13}C-^{15}N coupling data, requires that carbons directly attached to nitrogen exhibit significantly larger coupling constants than carbons further removed. Although ^{13}C-^{15}N couplings have been established for a variety of structurally distinct molecules,[4,16] examination of the recent literature reveals that only a few aromatic and heteroaromatic molecules have been studied. Representative ^{13}C-^{15}N coupling data for aniline,[17] 2-methoxyaniline,[18] nitrobenzene,[19] pyridine,[20] its hydrochloride[20] and *N*-oxide,[21] quinoline and its hydrosulfate,[22] pyridone,[23] and pyrrole[21] are shown in Figure 3. It is quite evident from these data that significant variations in J_{CN} exist for structurally related molecules; thus, it is not surprising that attempts to relate J_{CN} to structural features and to degree of hybridization at carbon and nitrogen have had only limited success.[16,24,25]

In view of the above observations and the lack of available coupling data for phenyl-substituted amides and quinolones, two ^{15}N-labeled models were synthesized. [^{15}N]Acetoacetanilide (**3**) was prepared from [^{15}N]aniline (97 atom % ^{15}N) and diketene;[26] 1,4-dimethyl-2-[^{15}N]quinolone (**6**) was derived from labeled **3** by cyclization to 4-methyl-2-[^{15}N]quinolone[27] followed by methylation with dimethyl sulfate.

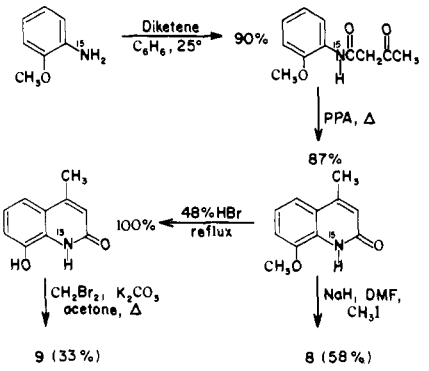


Figure 4. Preparation of ^{15}N-labeled model compounds (**8** and **9**) from [^{15}N]*o*-anisidine.

Carbon–nitrogen coupling data extracted from ^{13}C NMR spectra of labeled **3** and **6** are shown in Figure 1. The significantly larger coupling between nitrogen-15 and nitrogen-bonded aromatic carbons serves to distinguish those carbons from ones further removed and, thus, should readily permit identification of the nitrogen-bonded carbons in ^{15}N-labeled nybomycin.

[3,11-$^{15}N_2$]Nybomycin was obtained by growing *Streptomyces* sp. D-57 for 20 days on starch–nitrate medium containing sodium [^{15}N]nitrate (99 atom % ^{15}N, 4 g/L) as the sole nitrogen source. The crude nybomycin produced was isolated, converted to the *n*-butyrate, then purified. Mass spectral analysis indicated an average enrichment of 96% at each nitrogen atom.

The ^{13}C NMR spectrum of ^{15}N-labeled **2** (Figure 2) shows nine of the 20 signals split into doublets. Magnitudes of J_{CN} for all coupled carbons of **2** are given in Figure 1. Only three of the six resonances due to the central ring carbons are coupled. The two signals possessing large ^{13}C–^{15}N couplings can be assigned to C-11a and C-12a (125.6 ppm, $^1J_{CN}$ = 15.8 Hz and 132.2 ppm, $^1J_{CN}$ = 12.2 Hz), while the remaining peak is obviously due to C-12 (135.5 ppm, $^2J_{CN}$ = 3.6, $^2J_{CN'}$ < 0.7 Hz). Couplings for the six nonaromatic resonances (pyridone rings, *N*-methyl, *N*-methylene) correspond quite well to the analogous couplings in models **3** and **6**, and to established literature values;[4,16] these couplings are also quite similar to those obtained by Vining et al. in their structural and biosynthetic study of ^{15}N-labeled tenellin,[28] a pyridone-based fungal metabolite.

Specific assignments to individual carbons C-11a (125.6 ppm) and C-12a (132.2 ppm) are then made on the basis of calculated resonance values from the model study. However, it was of practical interest to ascertain if the significantly different magnitudes of $^1J_{CN}$ for C-11a and C-12a could be used to assign these resonances independently. Although no universal correlations have been established for variation of J_{CN} with degree of hybridization or with subtle structural differences,[16,25] certain trends have been demonstrated within a series of closely related compounds. For example, a recent study of saturated amides has shown that J_{CN} increases slightly with strain at the carbonyl substituent;[29] thus, differences between the two quinolone rings in **2** (compare C-4 with C-10 and C-5 with C-9) could possibly reflect a difference in hybridization between the two nitrogens or an increase in strain at N-3 as a result of the fused oxazoline ring. Also, the observation of a doublet for C-12 might be explained by assuming an abnormally large ortho coupling (3.6 vs. <0.7 Hz) to N-3 as a consequence of added strain; if $^2J_{CN}$ of each nitrogen were of comparable magnitude, a triplet would have been observed for C-12 instead of a doublet (Figure 2).

To investigate the dependence of J_{CN} on structure, ^{15}N-labeled **8** and **9** were studied. Both compounds were synthesized from 2-methoxy[^{15}N]aniline by the established methods[30] outlined in Figure 4. Labeled 2-methoxyaniline was obtained by Hofmann rearrangement of 2-methoxy[^{15}N]-benzamide which, in turn, was derived from [^{15}N]ammonia and 2-methoxybenzoyl chloride. Examination of the proton-decoupled ^{13}C NMR spectra of ^{15}N-labeled **8** and **9** afforded ^{13}C–^{15}N coupling data shown in Figure 1.

The ^{13}C–^{15}N coupling constants for outer pyridone ring carbons of **8** (C-1′, C-2, and C-3) and **9** (C-2, C-4, and C-5) agree quite well with the corresponding carbons in **2** (C-11′, C-10, and C-9; C-2, C-4, and C-5), but the magnitudes for nitrogen-bonded aromatic carbons in **8** and in **9** are identical and, therefore, do not permit distinction between C-11a and C-12a in **2**. It should be noted, however, that the abnormally large ortho coupling observed for C-12 ($^2J_{CN}$ = 3.6 Hz) in **2** is also found in ^{15}N-labeled **9** (C-9, $^2J_{CN}$ = 3.3 Hz). Apparently, certain subtle structural differences between C-11a and C-12a in nybomycin *n*-butyrate are not reflected in the models studied; this may be due to the absence of additional aromatic substituents at C-6 and C-7 in **8** and at C-7 and C-8 in **9** or to unique properties (strain, hybridization) associated with the fused, four-ring system of **2**.

The order in magnitudes of $^1J_{CN} \gg {}^3J_{CN} > {}^2J_{CN}$ for aromatic carbons of models **3**, **4**, **6**, **8**, **9**, and **10** is identical with that for **16**, **17**, **19**, and **21**, but differs sharply from that in pyridine (**15**) and quinoline (**18**). The abnormal behavior of pyridine and quinoline has been attributed to coupling contributions from sources other than the normal Fermi contact mechanism, which arise from interactions of the nitrogen lone pair.[20,22] Removal of the lone pair by protonation or quaternization, i.e., conversion of **15** to **16** or **17** and of **18** to **19**, gives values for $^1J_{CN}$ in agreement with those anticipated from theoretical considerations.[21,25] The lone pair dependence on coupling is also quite evident by the marked geometrical dependence of $^2J_{CN}$ on the orientation of the nonbonded electrons on nitrogen for C-8 of quinoline (**18**, Figure 4), that is, $^2J_{CN}$ = 9.3 Hz. A similar but less dramatic geometrical dependence also appears to function in aniline (**12**) and 2-methoxyaniline (**13**) ($^1J_{CN} \gg {}^2J_{CN} > {}^3J_{CN}$). Note that conversion of each to its corresponding acetoacetanilide (**3** and **4**, respectively) alters $^2J_{CN}$, i.e., $^3J_{CN} > {}^2J_{CN}$. The discrepancies between the reported ^{13}C–^{15}N couplings of pyridone (**20**) and those of model quinolones **6**, **8**, **9**, and **10** are difficult to explain. It is conceivable that the introduction of substituents on pyridone could alter the magnitudes of J_{CN}, but the very low values for C-6 (2.5 Hz) and for C-3 (<0.5 Hz) may be a consequence of chemical shift misassignments.[31]

Finally, examination of long range ^{13}C–^{15}N couplings in model acetoacetamides and quinolones reveals that meta couplings ($^3J_{CN}$) are decreased by substitution on the carbon between the meta carbon and the nitrogen bearing carbon (compare **3** with **4**, **6** with **8**, and **6** with **9**). This conclusion is also supported by the absence of secondary splitting of C-11a and C-12a in **2** (i.e., absence of a doublet of doublets (Figure 2)), by the decrease in both $^2J_{CN}$ (C-2) and $^3J_{CN}$ (C-3) of aniline upon substitution with a 2-methoxyl function (**12** to **13**), and by the difference in magnitude of $^3J_{CN}$ for C-5 and C-7 in **19**. These observations may prove quite useful in aiding chemical shift assignments of multisubstituted, nitrogen containing aromatic hydrocarbons.

Conclusions

^{13}C–^{15}N coupling data have proved to be a valuable adjunct to model compound studies in assigning the ^{13}C NMR spectrum of the antibiotic nybomycin. Application of this method to aid in assignment of the spectra of other nitrogen-containing natural products (fungal metabolites, alkaloids, and certain antibiotics) will depend primarily on the ability to obtain significantly enriched compounds in order to observe carbon–

nitrogen coupling constants. Future correlations of ^{13}C-^{15}N couplings with structural variations should enhance the utility of this method by providing additional information for structural and ^{13}C NMR resonance assignments.

Experimental Section

General. Melting points were taken on a Thomas–Hoover capillary melting point apparatus; all are uncorrected and given in degrees Centigrade. Microanalyses were obtained by Mr. J. Nemeth and his associates; all data are within 0.25% of the calculated values.

Proton magnetic resonance (^1H NMR) spectra were obtained with a Varian spectrometer, Model A-60A. Proton-decoupled carbon magnetic resonance (^{13}C NMR) spectra were obtained by Mr. S. Silber on a Varian spectrometer, Model XL-100-15, interfaced with a Digilab NMR-3 data system (256K disk) operating at 25.2 MHz. ^{13}C NMR samples were run in 12-mm tubes (total volume, 2.0–2.5 mL) at a probe temperature of 35 °C with an internal ^2H lock (deuteriochloroform). Spectra were obtained in 2–12 h using the following typical conditions: sweep width 5882 Hz, pulse width 15–20 μs (flip angle ~45°), acquisition time 1–1.5 s, pulse delay 0–0.025 s, spin decoupler offset 45 300 Hz, noise bandwidth 2 KHz, and a sampling of 16K data points. At 16K points the system provides a frequency resolution of 0.7 Hz. The conditions employed for off-resonance decoupled spectra were essentially the same except the spin decoupler was offset 44 300 Hz.

Proton-coupled ^{13}C NMR spectra, proton-decoupled spectra of ^{15}N-labeled compounds, the modified CW off-resonance spectrum of **6**, and specific proton-decoupled spectra were run on a Jeol FX-60 FT NMR spectrometer, operating at 15.0 MHz in the ^{13}C mode and at 59.80 MHz in the ^1H mode. Samples were run in 10-mm tubes at a probe temperature of ~30 °C with an internal ^2H lock (deuteriochloroform). Typical conditions were as follows: pulse width 10 μs (flip angle ~45°), pulse delay ~250 μs, spin decoupler offset 47 800 Hz, noise bandwidth 1 KHz, and sampling of 8192 data points. The sweep width, generally 1700–1900 Hz, was chosen to provide a frequency resolution of >0.5 Hz. ^{13}C–^{15}N coupling constants, the average value of at least two runs, are accurate within ±0.5 Hz. For the modified CW off-resonance spectrum of **6**, the decoupler offset was placed ca. ~750 Hz upfield from the aromatic proton resonances of **6**.

^{13}C NMR spectral data for all compounds except 2-methoxyaniline (**13**) are provided in Figure 1 of the text. Peak assignments are consistent with multiplicities observed in off-resonance decoupled spectra. NMR shift data are given in parts per million downfield from internal Me_4Si. The abbreviations used are s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. Coupling constants J, J_{CN}, and J_{NH} refer to proton–proton, carbon-13–nitrogen-15, and nitrogen-15–proton interactions, respectively.

Low-resolution mass spectra were obtained by Mr. J. Wrona on a Varian MAT spectrometer, Model CH5. For ^{15}N-enrichment determinations, isotope-ratio (flat-topped peak) mass spectral scans (10–15 scans per sample) were obtained at the lowest possible electron voltage (~8–10 eV) in order to minimize contributions from the M − 1 peak. Intensity data for both enriched and unenriched compounds were normalized relative to the intensity of the molecular ion (=1.00), then averaged and adjusted as outlined by Biemann[32] to provide mole percent of each labeled species.

Nybomycin *n*-Butyrate (2). Nybomycin (100 mg, 0.336 mmol) was dissolved in 96% sulfuric acid (2 mL) and *n*-butyric acid (10 mL, 109 mmol) was added with stirring. The mixture was heated at 130 °C for 2.5 h, then cooled, poured over ice water, and extracted with chloroform (five 30-mL portions). The chloroform layer was washed successively with 5% aqueous sodium carbonate (five 30-mL portions) and water (50 mL), dried over magnesium sulfate, and filtered. Removal of chloroform under reduced pressure gave the light yellow butyrate ester (99 mg). Column chromatography on silica gel (35 g) using benzene–2-propanol (97:3) as eluent removed two minor impurities. Evaporation of solvent afforded nybomycin butyrate as a light yellow powder (72 mg, 59%): mp 203–204 °C (lit.[33] 203–204 °C); ^1H NMR ($CDCl_3$) δ 0.99 (3 H, t), 1.74 (2 H, sextet), 2.45 (2 H, t), 2.47 (3 H, d, J = 1.2 Hz), 3.92 (3 H, s), 5.38 (2 H, d, J = 1.0 Hz), 6.39 (2 H, s), 6.46 (1 H, q, J = 1.2 Hz), 6.70 (1 H, t, J = 1.0 Hz), 7.39 (1 H, s).

2-Methoxyacetoacetanilide (4). The procedure of Williams and Krynitsky[26] provided 2-methoxyacetoacetanilide. Recrystallization from cyclohexane–benzene (1:1) produced white needles: mp 83–84.5 °C (lit.[34] 87 °C); ^1H NMR ($CDCl_3$) δ 2.30 (3 H, s), 3.59 (2 H, s), 3.89 (3 H, s), 6.78–7.20 (3 H, m), 8.17–8.45 (1 H, m), 9.1 (1 H, br s).

2,6-Diacetoacetamidoanisole (5). 2,6-Diacetoacetamidoanisole was prepared by the method of Forbis and Rinehart.[30] The crude substituted anisole was purified by passage over a short column of silica gel using chloroform. Removal of solvent and recrystallization from 2-propanol provided pale yellow needles: mp 129.5–131.5 °C (lit.[30] 126–128 °C).

1,4-Dimethyl-2-quinolone (6). 4-Methyl-2-quinolone (880 mg, 5.5 mmol), prepared by the method of Lauer and Kaslow,[27] was dissolved in 5% aqueous sodium hydroxide solution (45 mL) by heating to ~80 °C. Dimethyl sulfate was then added in small portions to the well stirred, hot solution until pH 3–4 was obtained. Upon cooling, the solution produced crystals of the desired quinolone (700 mg, 4.05 mmol, 74%). Recrystallization from water gave **6** as white needles: mp 128–129 °C (lit.[35] 130–132 °C); ^1H NMR ($CDCl_3$) δ 2.41 (3 H, d), 3.68 (3 H, s), 6.56 (1 H, br s), 7.05–7.80 (3 H, m).

4-Acetoxymethyl-1-methyl-2-quinolone (7). The procedure of Hasegawa[36] was used to provide 4-acetoxymethyl-2-quinolone in 68% yield: mp 213–215 °C (lit.[36] 212–215 °C). A mixture of 4-acetoxymethyl-2-quinolone (566 mg, 2.6 mmol), methyl iodide (3.69 g, 26 mmol), anhydrous potassium carbonate (1.0 g, 7.8 mmol), and acetone (40 mL) was heated at reflux for 24 h, cooled, and filtered. Solvent was removed to afford a white residue, which was taken up in chloroform and dried over magnesium sulfate. Filtration and removal of solvent gave a white solid (375 mg, 1.56 mmol, 60%). Two crystallizations from ethanol provided **7** as white needles: mp 117–117.5 °C; ^1H NMR ($CDCl_3$) δ 2.19 (3 H, s), 3.70 (3 H, s), 5.32 (2 H, d, J = 1 Hz), 6.78 (1 H, t, J = 1 Hz), 7.10–7.73 (4 H, m); mass spectrum *m/e* 231 (M^+, 45), 189 (100), 188 (42), 160 (38), 144 (33), 43 (36). Anal. ($C_{13}H_{13}NO_3$) C, H, N.

6-Methyl-2*H*,4*H*-oxazolo[5,4,3-*ij*]quinolin-4-one (9). The method of Forbis and Rinehart[30] was used, but modified by increasing the mole ratio of methylene bromide to 8-hydroxy-4-methyl-2-quinolone from 7.6 to 19.3. An improved yield of **9** was obtained (47%): mp 196.5–197.5 °C (lit.[30] 193–195 °C); ^1H NMR ($CDCl_3$) δ 2.34 (3 H, d, J = 1.2 Hz), 6.23 (2 H, s), 6.43 (1 H, q, J = 1.2 Hz), 6.77–7.12 (3 H, m).

1,4-Dimethyl-8-methoxy-2-quinolone (8). 8-Methoxy-4-methyl-2-quinolone (**10**) (1.9 g, 0.010 mol), prepared by the method of Forbis and Rinehart,[30] was added in three portions to a suspension of sodium hydride (0.3 g, 0.013 mol) in dimethylformamide (40 mL). After 30 min at 25 °C, methyl iodide (2.5 g, 0.018 mol) in 16 mL of dimethylformamide was introduced. The mixture was stirred for 2 h, then heated at 60 °C for 2 hr. After cooling, a few drops of water were added and the solvent was removed under reduced pressure. The residue was treated with water (40 mL), then extracted with chloroform (four 60-mL portions); the combined chloroform extract was dried over magnesium sulfate and filtered. Removal of solvent gave a mixture (2.3 g) of **8** and 2,8-dimethoxy-4-methylquinoline. Column chromatography over silica gel using chloroform permitted separation of the *O*-methylated product (0.3 g, 15%) from the desired **8** (1.6 g, 79%). Recrystallization (twice) from cyclohexane afforded pure **8** as white needles; mp 69–70.5 °C; IR ($CHCl_3$) 1650 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.35 (3 H, d), 3.84 (3 H, s), 3.86 (3 H, s), 6.52 (1 H, br s), 6.98–7.41 (3 H, m); mass spectrum *m/e* 203 (M^+, 100), 188 (86), 173 (26), 160 (30). Anal. ($C_{12}H_{13}NO_2$) C, H, N.

9-Methoxy-1,4,5,8-tetramethyl-2,7-dioxo-1,2,7,8-tetrahydro-1,8-diazaanthracene (11). Demethylenedeoxynybomycin (700 mg, 2.6 mmol) obtained from deoxynybomycin,[33] anhydrous potassium carbonate (4.5 g, 32.5 mmol), and methyl iodide (50.2 g, 355 mmol) was heated at reflux in dimethylformamide (60 mL) for 24 h. After cooling, the reaction mixture was concentrated to approximately 25 mL under reduced pressure, then poured into an aqueous sodium thiosulfate solution. The white precipitate which formed was collected and dissolved in chloroform; the solution was dried over magnesium sulfate and filtered, and solvent was removed to yield a pink solid (~1 g). Column chromatography over silica gel with chloroform–ethanol (97:3) provided two compounds (~400 mg each). The faster moving component was twice recrystallized from ethanol to afford white needles of **11**: mp 304–305 °C dec; IR (KBr) 1650 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.53 (6 H, d, J = 1.5 Hz), 3.52 (3 H, s), 3.87 (6 H, s), 6.59 (2 H, q, J = 1.5 Hz), 7.70 (1 H, s); mass spectrum *m/e* 298 (M^+, 81), 283 (100), 268 (24), 255 (27), 240 (39). Anal. ($C_{17}H_{18}N_2O_3$) C, H, N.

[^{15}N]**Acetoacetanilide (3).** Treatment of 1.0 g (10.7 mmol) of [^{15}N]aniline (Prochem/BOC Ltd., London, 97.0 atom % ^{15}N) with 0.9 g (10.7 mmol) of diketene, using the procedure of Williams and Krynitsky,[26] afforded crude acetoacetanilide. Recrystallization from ethanol–water (1:1) resulted in white needles of the desired [^{15}N]-acetoacetanilide (0.51 g). An additional 0.95 g of **3** (total yield, 77%) was recovered by concentration of the mother liquor and column chromatography over silica gel using chloroform as eluent. [^{15}N]-Acetoacetanilide had mp 79.5–80.0 °C (lit.[26] 84–85 °C); ^{1}H NMR ($CDCl_3$) δ 2.25 (3 H, s), 3.50 (2 H, br s), 6.90–7.60 (5 H, m), 8.99 (1 H, d, J_{NH} = 89 Hz). Weak absorptions of low intensity at 1.88 and 4.93 were attributed to the enol form.

1,4-Dimethyl-2-[^{15}N]quinolone (6). Labeled 4-methyl-2-quinolone was prepared by modification of the procedure of Lauer and Kaslow.[27] [^{15}N]Acetoacetanilide (1.0 g, 5.6 mmol) was added portionwise with stirring to concentrated sulfuric acid (5 mL) at 72 ± 3 °C (bath temp). When addition was complete (30 min), the bath temperature was raised to 95 °C and maintained for an additional 30 min. The mixture was cooled to 50 °C and poured into ice–water. The cream precipitate was collected, washed with water, and dried (0.64 g); extraction of the filtrate with chloroform and subsequent removal of solvent produced an additional 0.09 g of crude 4-methyl-2-[^{15}N]quinolone (total yield, 81%).

The crude labeled quinolone (730 mg, 4.56 mmol) was converted to labeled **6** in 80% yield using the procedure described above for unlabeled **6**. Recrystallized ^{15}N-labeled **6** had mp 127–128 °C (lit.[35] 130–132 °C); ^{1}H NMR ($CDCl_3$) δ 2.42 (3 H, d, J = 1.4 Hz), 3.63 (3 H, d, J_{NH} = 1.4 Hz), 6.52 (1 H, br s), 7.02–7.68 (4 H, m). Isotope-ratio mass spectra (flat-topped peaks) showed 3.7% unlabeled and 96.3% labeled quinolone (average enrichment 96.3%).

[3,11-$^{15}N_2$]Nybomycin *n*-Butyrate (2). All biological manipulations were performed under aseptic conditions. Media and equipment were sterilized by use of a standard autoclave (120 °C, 15 psi, 20 min). Sterile slants of yeast–malt extract agar (composed of yeast extract, 4 g; malt extract, 10 g; dextrose, 4 g; agar, 20 g; distilled water to 1 L) were inoculated with *Streptomyces* sp. D-57 (soil stock from The Upjohn Co.). Slants were incubated at 30 °C and, by the seventh day, sporulation was observed as indicated by the gray fuzzy appearance of the culture. Sterile water (3 mL) was added to each slant, the spores dislodged by means of a wire loop, and the agar broken up. Each slant was then transferred to an Erlenmeyer flask (500 mL, fitted with a cotton plug) containing 100 mL of sterile starch–nitrate medium (composed of soluble starch, 10 g; sodium nitrate, 4 g; potassium dihydrogen phosphate, 0.7 g; potassium monohydrogen phosphate, 4 g; magnesium sulfate, 2 g; sodium chloride, 3 g; "trace element mixture," 1 mL; distilled water to 1 L. The trace element mixture consisted of: zinc sulfate, 1.15 g; ferrous sulfate, 1.11 g; copper sulfate, 0.64 g; manganous chloride, 0.79 g; distilled water to 100 mL. The organism was permitted to grow 4 days in a rotary incubator–shaker (270 rpm) at 30 °C. On the fourth day, seed medium (5 mL per flask) was added to three production flasks (1 L). Each production flask contained starch–nitrate medium (333 mL) prepared with sodium [^{15}N]nitrate (Koch Isotopes, isotopic purity 99.2%). Flasks were incubated under the same conditions as seed flasks and after 20 days growth, mycelia were harvested and dried (100 °C, 6 h, 0.05 Torr); total nybomycin content of the dry mycelia (2.4 g) was 127 mg by bioassay.[37] Extraction of the mycelia with boiling dimethyl sulfoxide (three 100-mL portions) and removal of solvent under reduced pressure afforded a brown, partially solidified oil which was treated with 1% aqueous sodium versenate (50 mL) at 90 °C for 30 min, cooled, and filtered. The crude antibiotic was washed with cold water and acetone, then dried (60 °C, 12 h, 0.05 Torr).

Crude labeled nybomycin (134 mg) was converted to the *n*-butyrate by the procedure described above. [3,11-$^{15}N_2$]Nybomycin *n*-butyrate (66 mg) had mp 203–204 °C (lit.[33] 203–204 °C); ^{1}H NMR ($CDCl_3$) δ 0.99 (3 H, t), 1.74 (2 H, sextet), 2.43 (2 H, t), 2.46 (3 H, d, J = 1 Hz), 3.87 (3 H, d, J_{NH} = 1.3 Hz), 5.30 (2 H, br s), 6.30 (2 H, s), 6.40 (1 H, br s), 6.65 (1 H, br s), 7.30 (1 H, s). Isotope-ratio mass spectra (flat-topped peaks) indicated 1.9% unlabeled, 4.8% monolabeled, and 93.3% dilabeled nybomycin butyrate (average enrichment of 95.7% at each nitrogen atom).

2-Methoxy[^{15}N]benzamide. A 1-L gas bulb, containing [^{15}N]-ammonia (ca. 0.045 mol, 99 atom %, Koch Isotopes), was cooled in a dry ice–acetone bath. The seal was broken and replaced with a rubber septum. Triethylamine (4.65 g, 6.4 mL) was introduced to the vessel via a syringe followed by freshly distilled, ethanol free chloroform (60 mL). Finally, 2-methoxybenzoyl chloride (7.6 g, 0.045 mol), prepared from oxalyl chloride and sodium 2-methoxybenzoate,[38] was introduced as a chloroform solution (7.6 g in 15 mL). The flask was warmed slowly to 25 °C over 1 h, then agitated an additional hour at 25 °C. The clear solution was quantitatively transferred to a larger flask and the solvent was removed under reduced pressure. The resulting white solid was suspended in 50 mL of ice–water to solubilize the triethylamine hydrochloride by-product; suction filtration gave the crude benzamide, which was washed with 40 mL of cold water, then dried. Crude 2-methoxy[^{15}N]benzamide (6.0 g) was crystallized from benzene to afford white needles (5.2 g, 76%): mp 125–127 °C (lit.[39] 128 °C); ^{1}H NMR ($CDCl_3$) δ 3.91 (3 H, s), 6.87 (1 H, dd, J = 4, J_{NH} = 88 Hz), 6.90–7.70 (3 H, m), 7.75 (1 H, dd, J = 4, J_{NH} = 88 Hz), 8.15–8.36 (1 H, m).

2-Methoxy[^{15}N]aniline (13). The procedure of Buck and Ide[40] was adapted for this conversion. A solution of sodium hypochlorite was prepared by bubbling chlorine gas (2.5 g, 0.034 mol) into a cold sodium hydroxide solution (8.5 g in 53 g of water and 32 g of ice). To this solution was added 2-methoxy[^{15}N]benzamide (5.15 g, 0.034 mol); the suspension was stirred and heated slowly (15–20 min) to 50–55 °C under nitrogen. Heating was gradually continued until the dark mixture reached 70 °C; after 1 h, an aqueous solution of sodium hydroxide (12.8 g in 13 mL) was added and the mixture was heated to 80 °C. After 1 h at 80 °C, the two-phase system was cooled, then extracted with ether (four 100-mL portions). The combined ether extract was dried over magnesium sulfate and filtered. Removal of solvent produced a dark oil (3.6 g), which was distilled at ~2 Torr to provide 2-methoxy[^{15}N]aniline (3.14 g, 75%) as a clear, slightly viscous oil: ^{1}H NMR (CCl_4) δ 3.60 (3 H, s), 3.61 (2 H, br d, J_{NH} = 75 Hz), 6.35–6.75 (4 H, m); ^{13}C NMR ($CDCl_3$) 54.9 (OCH_3), 110.0 (C-3), 114.4 (J_{CN} = 2.6 Hz, C-6), 117.6 (C-4), 120.5 (J_{CN} = 1.3 Hz, C-5), 135.8 (J_{CN} = 11.4 Hz, C-1), 146.6 ppm (J_{CN} = 0.9 Hz, C-2).

2-Methoxy[^{15}N]acetoacetanilide (4). Labeled **4** was prepared in 90% yield from ^{15}N-labeled **13** by the procedure of Williams and Krynitsky.[26] ^{15}N-labeled **4** had the following physical properties: mp 83–85 °C (lit.[34] mp 89 °C); ^{1}H NMR ($CDCl_3$) δ 2.20 (3 H, s), 3.51 (2 H, br s), 3.81 (3 H, s), 6.67–7.10 (3 H, m), 8.10–8.40 (1 H, m), 9.07 (1 H, d, J_{NH} = 92 Hz).

8-Methoxy-4-methyl-2-[^{15}N]quinolone (10). The procedure of Forbis and Rinehart[30] provided ^{15}N-labeled **10** from ^{15}N-labeled **4** in 87% yield: mp 188–190 °C (lit.[41] mp 188–190 °C); ^{1}H NMR ($CDCl_3$) δ 2.45 (3 H, d, J = 1.2 Hz), 3.99 (3 H, s), 6.56 (1 H, d, J = 1.2 Hz), 6.89–7.39 (3 H, m), 9.38 (1 H, br s).

1,4-Dimethyl-8-methoxy-2-[^{15}N]quinolone (8). ^{15}N-labeled **10** (1.2 g, 0.006 mol) was methylated as described above for unlabeled **8** to provide a mixture (1.1 g) of ^{15}N-labeled **8** and 2,8-dimethoxy-4-methyl[^{15}N]quinoline, which was separated by preparative TLC on silica gel using chloroform–ethanol (98:2). Pure ^{15}N-labeled **8** (0.75 g, 58%) had mp 67–68 °C (mp unlabeled, 69–70.5 °C). Isotope-ratio mass spectral analysis indicated an average ^{15}N enrichment of 90.1%.

8-Hydroxy-4-methyl-2-[^{15}N]quinolone. Treatment of ^{15}N-labeled **10** (2.2 g, 0.0116 mol) with 48% hydrobromic acid according to the method of Forbis and Rinehart[30] gave 8-hydroxy-4-methyl-2-[^{15}N]quinolone (2.05 g) in 100% yield, mp 253–255 °C (lit.[30] 248–250 °C).

6-Methyl-2*H*,4*H*-[^{15}N]oxazolo[5,4,3-*ij*]quinolin-4-one (9). ^{15}N-labeled **9** was synthesized from labeled 8-hydroxy-4-methyl-2-quinolone in 33% yield by the above procedure for unlabeled **9**. ^{15}N-labeled **9** had mp 193–195 °C (lit.[30] 193–195 °C). Isotope-ratio mass spectral analysis showed an average ^{15}N enrichment of 90.2%.

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Nucleic Acid Related Compounds. 26. A "Geometry-Only" Method for Determining the Anomeric Configuration of Nucleosides Based on the H-1′ NMR Signal of Cyclic α and β 3′,5′-Mononucleotides[1]

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Abstract: Conversion of ribonucleosides to their cyclic 3′,5′-monophosphates (3′,5′-cNMP's) provides a derivative with a trans-fused six- to five-membered ring system. The spin–spin coupling of the anomeric proton (H-1′) with H-2′ of these conformationally rigid cyclic mononucleotides is now uniquely controlled by the geometry of the fused molecular system. The anomeric proton of β anomers gives rise to a sharp singlet [$J_{1'-2'}$(trans) $\lesssim$ 0.7 Hz] whereas the corresponding ^1H NMR signal for α anomers appears as a doublet [$J_{1'-2'}$(cis) $\gtrsim$ 3.5 Hz] in over 200 examples with *no observed exception.* Some 15 additional examples of arabinofuranosyl nucleoside and cyclonucleoside 3′,5′-cNMP's also exhibit the expected anomeric proton doublet [$J_{1'-2'}$(cis) = 6–7 Hz]. Limitations of and exceptions to previous approaches to the determination of anomeric configuration of ribofuranosyl compounds are discussed.

Previous approaches to the determination of anomeric configuration of ribonucleosides have employed chemical reactions, chiroptical methods, and NMR spectroscopy. However, all of these methods have involved specific structural requirements in the base moiety and/or conformational effects which have proven to be empirically useful in many cases but which are not fully understood or predictable a priori. Certain of these methods are applicable only if both anomers are available for comparison.

Hudson's rules of isorotation[3] for carbohydrate derivatives