JOURNAL OF combinatorial CHEMISTRY

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J. Comb. Chem., **2001**, 3 (5), 434-440• DOI: 10.1021/cc000101t • Publication Date (Web): 28 June 2001

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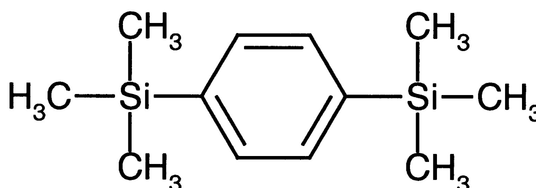

BTMSB = 1,4-bis(trimethylsilyl)benzene

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Characterization of Small Combinatorial Chemistry Libraries by ^{1}H NMR. Quantitation with a Convenient and Novel Internal Standard

Vittorio Pinciroli, Roberto Biancardi, Nicoletta Colombo, Maristella Colombo, and Vincenzo Rizzo*

Pharmacia Corporation, Viale Pasteur 10, I-20014 Nerviano (MI), Italy

Received November 27, 2000

A novel silane standard, 1,4-bis(trimethylsilyl)benzene (BTMSB), is introduced for the generic quantitation of small organic molecules in DMSO-d_6 solution by ^{1}H NMR. This standard is an easily weighable solid and is stable for at least 1 month in DMSO solution, and its ^{1}H NMR spectrum contains a strong singlet in a region usually free of signals. With a set of certified standards, concentration determination with about 2% precision and accuracy is verified after solution preparation with fully automated procedures, thus making very effective the characterization of small combinatorial chemistry libraries for identity and purity when combined with other physicochemical or biochemical tests. As an example, for a set of about 400 compounds, results of ^{1}H NMR characterization are compared to the more customary LC−UV−MS method. NMR and MS data agree for identity on the vast majority of cases (84% positive and 5% negative), whereas the remaining cases (11%) are marked as highly impure only after NMR spectra analysis. Most importantly, determination of concentration rather than that of relative purity appears the right choice for a correct evaluation of biochemical potency.

Introduction

Structural confirmation and purity estimation of new synthetic molecules are necessary steps in the exploitation of these compounds. In particular, an accurate knowledge of absolute purity (strength) or concentration in solution is important to carefully evaluate pharmacological activity (potency). Analytical characterization of the large number of products coming from combinatorial synthesis is usually based on HPLC−UV−MS,[1] but the variability of UV and MS response factors makes it a questionable choice for purity or concentration determination. New universal detectors are being investigated for the rapid quantification of small organic molecules, the more promising ones being ELSD[2] (evaporative light scattering detector) and CLND[3,4] (chemiluminescent nitrogen detector). However, these new tools suffer from a rather high quantitation error (10−20%) and each one has specific limitations.

Before the advent of combinatorial chemistry, NMR was considered the most suitable technique for analytical characterization of new entities because of the wealth of structural information contained in the spectrum. Under appropriate conditions NMR is also a universal quantitative detector because the response factor is proportional to the number of nuclei associated with a given signal independent of the molecular species. Consequently, NMR is particularly convenient for purity measurements because quantitation is in principle possible using a single reference compound without the need of a well-characterized reference standard for every sample. Moreover, ^{1}H NMR is capable of proving purity with 1−2% accuracy, as we show with extensive validation tests in this paper, and it has been demonstrated recently in three different reports.[5−7] Drawbacks of NMR are the inherent low sensitivity and the comparatively inadequate rapidity. Modern high-field NMR spectrometers equipped with high signal-to-noise probes, sample changers, and fully automated data collection are capable of producing at least four good-quality spectra, suitable for quantitative analysis, per hour with 3−4 mM solutions (<1 mg of compound) on a 24 h per day basis. Further reduction of sample need is nowadays possible with special dedicated flow probes.[8] Nevertheless, spectra interpretation is still largely a “manual” process and is the rate-limiting step of the whole process. In our experience, a skilled operator dealing with simple molecules (as those synthesized in combinatorial libraries) may verify structure and purity of a small library (100−200 compounds) and file all data in an electronic database within a few days. This productivity could largely grow with the help of appropriate software tools for automatic spectra assignment; some commercial products are already available, and there is considerable effort in this area.[9]

In our discovery facilities, small libraries designed for lead optimization of specific structural templates are generally prepared by parallel synthesis and are made of single compounds. For the sake of providing full characterization of these libraries, we have developed a procedure that combines structural confirmation, purity characterization, assessment of solution properties (solubility, stability, etc.), and biological tests starting from a single DMSO stock solution of each compound. Since ^{1}H NMR was included in the test panel as a robust method to verify the identity of

* To whom correspondence should be addressed.

10.1021/cc000101t CCC: $20.00 © 2001 American Chemical Society
Published on Web 06/28/2001

compounds, we decided to investigate the feasibility of concentration determination by this technique. Robotic solution preparation accelerated the entire process.

The choice of an appropriate internal standard proved to be the most challenging step in the development of an appropriate quantitative NMR method. Ideally, the reference compound must be a highly pure and easily weighable solid that is soluble in the solvent of choice (DMSO in our case) and is stable for a long time under these conditions, and it should not react with any of the analyzed compounds. Moreover, it should have an intense singlet in the usually free region of the NMR spectrum. We tested several internal standards reported in the literature, but none of these performed satisfactorily (see Results for a discussion). Finally, among other commercially available trimethylsilyl derivatives, we discovered that 1,4-bis(trimethylsilyl)benzene (BTMSB) summed up all of these favorable properties and we decided to validate our method using this standard.

Results

1. Selection of the NMR Internal Standard. Silanes have been extensively used as reference standards in NMR; however, most simple silanes or siloxanes (i.e., TMS, hexamethyldisilane, hexamethyldisiloxane) are highly volatile liquids, and although this favors sample recovery after NMR analysis, their use for quantitative determinations in a routine environment proved to be very difficult in our hands. Nevertheless, Hamper et al.[10,11] have reported a method to measure polymer loading of combinatorial chemistry resins by cleavage with a solution of hexamethyldisiloxane (HMDS) in TFA/$CDCl_3$ (1:1) and acquisition of the quantitative ^{1}H NMR spectrum directly on the filtrate. These authors have also demonstrated that a ~10 mM HMDS solution in TFA/$CDCl_3$ (1:1) is stable for over 2 months. When HMDS was tested in DMSO-d_6, which is a good solvent for most organic compounds, evaporation loss during handling operations definitely interfered with the preparation of solutions having a predetermined concentration. Actually, Hamper et al.[10,11] established the HMDS concentration with NMR by adding a weighed amount of another reference compound. Moreover, the prepared HMDS solution (~0.5 mM) loses about 1% of its molar concentration per day (probably because of evaporation), as determined by periodically preparing NMR samples containing a known amount of acetanilide. Therefore, HMDS was judged to be inconvenient for our purposes.

Tetrakis(trimethylsilyl)silane is another silyl compound that is a solid and is less volatile, but unfortunately, we found it chemically unstable in DMSO solution. Other solid derivatives of trimethylsilane [i.e., sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d_4 and trimethylsilylpropanesulfonic acid] have been used as NMR standards, but these have significant residual resonances in an important region of the spectrum and are not stable in solution.[5] Maleic acid has been proposed as an internal standard,[5] but this compound, although stable in DMSO solution, is not chemically inert because of the reactive double bond and because of the free carboxyl function, which usually broadens signals of exchangeable protons (such as the residual water signal), sometimes making integration difficult. More recently, 2,5-dimethylfuran (DMFu) has been proposed as an ^{1}H NMR "traceless" internal standard,[12] though its resonances (~2.2 and 5.8 ppm) may in some cases overlap signals of the test compounds. We tested DMFu and observed that a properly conserved DMSO-d_6 solution is stable for at least 1 week, but because of high volatility (bp 92 °C), we encountered the same practical difficulties as in the case of HMDS solution preparation.

Finally, 1,4-bis(trimethylsilyl)benzene was found to meet at best our requirements. As a matter of fact, it is an easily weighable solid, soluble enough and stable in DMSO. It can be easily purified to 99.9% even though the commercial purity (96−98%) is acceptable for most purposes. Its ^{1}H NMR spectrum contains a strong singlet in a region that is usually free of signals (0.22 ppm). The disadvantage of not being removable from the sample is well balanced by its handiness, and in any case, it is not a big issue in our analytical scheme (just a total of 2 μmol of sample is used for NMR analysis and 0.2 μmol for MS). Furthermore, the preparation of a single DMSO-d_6 stock solution for all the other physicochemical or biochemical tests in our panel per se allows a large reduction in the sample consumption.

Moreover, because a separate solution is used for HPLC−UV−MS, the presence of an NMR internal standard does not affect routine purity determination. Anyway, BTMSB has a very low UV absorption above 230 nm; it is not detectable in the ESI ion source, and it displays a favorable chromatographic behavior because it is eluted at the maximum composition of the apolar phase (HPLC retention time is ≃8.2 min under our conditions; see Experimental Section), well after most of the compounds belonging to the structural classes we have so far analyzed in our laboratories.

2. Quantitative NMR Method Validation. 2.1. Stability of the Internal Standard Solution. A 0.556 ($^5/_9$) mM DMSO-d_6 solution of BTMSB was stored at room temperature in a desiccator for over a month and periodically assayed vs freshly prepared solutions of acetanilide. On each given day, three solutions of acetanilide were prepared and mixed in the usual volume ratio with the silane solution, thus making three independent samples (see Experimental Section). The silane strength was than assayed assuming 100% strength for acetanilide. The results are shown in Figure 1 as a function of the aging of the silane solution. Over more than a month no trend or time dependence is detected and the daily averaged values do not differ by more than 1% from the total average, which is very close to the assumed value (>99.9%). Accordingly, the solution can be used for up to 1 month of storage at room temperature.

2.2. Specificity. BTMSB resonances are two singlets at 0.22 and 7.48 ppm (longitudinal relaxation time T_1 is 2.4 and 2.2 s, respectively). Satellite signals (relative intensity of 2.4%) due to coupling with ^{29}Si are detected at 3.3 Hz on both sides of the 0.22 ppm peak. None of the compounds tested in our lab had resonances near the high-field silane singlet, and in our current experience this is very rarely observed. Overlap with the 7.48 ppm resonance is more commonly detected.

Specificity of a generic method can only be evaluated with a large set of compounds, and our experience confirms that

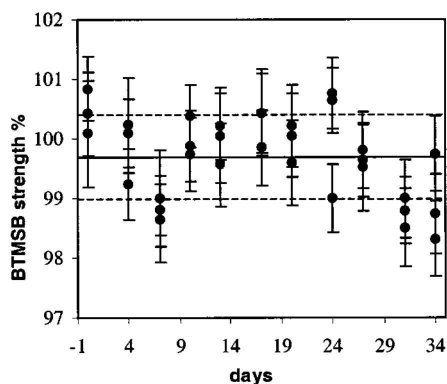


Figure 1. Stability test of a BTMSB solution over 34 days. Data are apparent strength of a silane stock solution prepared at day 0 and stored at room temperature. Values were obtained from assays with freshly prepared acetanilide solutions; each point is the result of an independent experiment. The solid and dotted lines represent the global average and standard deviation, respectively, whereas the *Y* error bars show the standard deviation of the integrated signals of each single sample.

Table 1. Precision of the NMR Quantitative Method

	operator 1		operator 2	
solution	purity, %	RSD, %	purity, %	RSD, %
1	98.14	0.95	98.68	0.58
2	99.33	0.99	98.91	0.35
3	97.66	0.71	98.27	0.53
4	97.98	0.90	98.43	0.38
5	96.83	0.76	97.05	0.59
6	100.67	0.93	101.08	0.48
average	98.43	1.36	98.74	1.32

isolated, cleanly integrable signals are easily found in the 400 MHz spectrum of simple organic molecules if the purity is relatively high. Nevertheless, the method can fail because of signal crowding when highly impure samples are tested.

With respect to traditional methods, NMR offers a notable sensitivity to even minor structural modifications (i.e., diastereoisomerism), and with the use of a standard, even species that do not have assignable proton NMR signals (salts, counterions, some solvents) are correctly accounted for in the determination of purity.

2.3. Precision and Accuracy. Precision of the assay is tested with the preparation of six different acetanilide solutions followed by data processing by two independent operators. Results are collected in Table 1, where the standard deviation derived from the multisignal averaging process is reported near each single datum; these are intrinsic measures of the precision in the NMR measurement alone (<1%). The last row of Table 1 collects the RSD (relative standard deviation) of averaging among the different solutions, which also contain the effect of solution preparation. In conclusion, reproducible values are obtained to better than 2% RSD. Correspondence of results obtained by different operators is good and definitely within the global precision limit.

For a test of accuracy nine different compounds of known purity, routinely used in our laboratories as standards for the quality control of active ingredients (acetanilide, cabergoline, caffeine, nicotinamide, daunorubicin hydrochloride, indobufene, nicergoline, penicillin G potassium salt, potassium hydrogen phthalate), were selected and assayed according to the procedure. Three different solutions were prepared for each compound, and the agreement with declared or assumed absolute purity (strength) was generally within ±2%. Figure 2 shows the frequency dependence of a normalized parameter

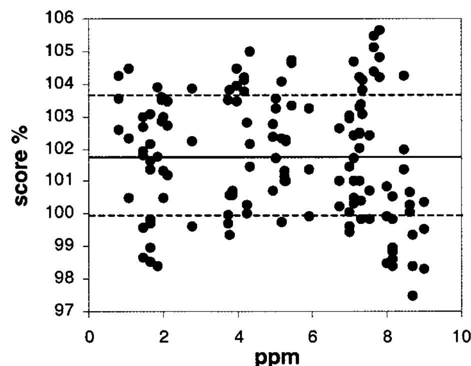


Figure 2. Accuracy test. The ratio of measured strength to certified strength is reported as score % for every integrated signal of nine different reference compounds vs chemical shift. The solid line indicates the mean of all experimental values, and dotted lines are shown at +1 SD and −1 SD. Multiple data refer to the three different solutions that were prepared for each standard.

$$\text{score \%} = 100 \times (\text{measured strength})/\text{certified strength}$$

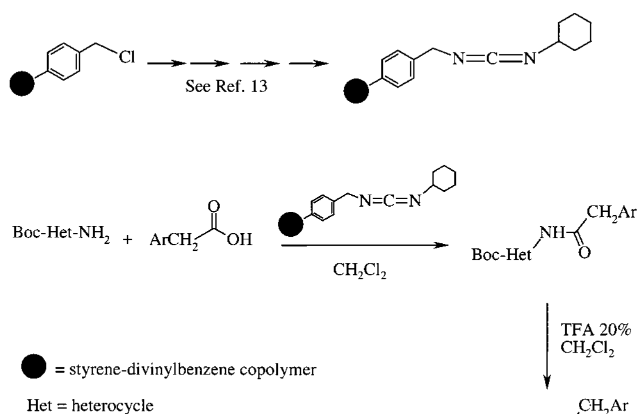
which permits comparison among different compounds. The value obtained for each integrated signal in the spectra of the above nine compounds is reported vs chemical shift; multiple data refer to different solutions. The figure clearly shows that data are distributed in a range of ±2% around an average value of about 102% without any apparent dependence on chemical shift. This confirms the good performance of the numerical correction for the nonlinear instrument response (see Experimental Section) and shows that errors probably originate from solution preparation and peak integration, two factors that should act randomly on the final results.

Data not reported in this paper show that both accuracy and precision can be improved to better than 1% if solutions are manually prepared with a micropipet instead of using a liquid handler.

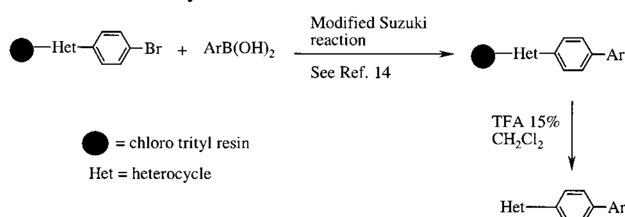
2.4. Linearity. A series of solutions were prepared by mixing volumes of the caffeine and acetanilide 10 mM solutions in a known ratio (1:9, 2:8, 4:6, 6:4, 8:2, 9:1). The regression lines of the found purity vs computed purity (acetanilide, $Y = 1.002X$, $r^2 = 0.9999$; caffeine, $Y = 1.020X$, $r^2 = 0.9998$) confirm the good linearity of the method. In the composition range 20−90% of this binary mixture, the precision of purity determination agrees with the general limits of the methods for both caffeine and acetanilide. Errors clearly increase at the 10% level. Nevertheless, the slope of the regression line only deviates from unity by 2% in one case and by 0.2% in the other.

3. Combinatorial Samples Data. In this paper we report data of three small libraries (libraries 1−3) of single compounds generated by parallel synthesis according to reaction paths shown in Schemes 1−3, respectively. The quantitative ^{1}H NMR spectrum of a typical member of library 3 is shown in Figure 3.

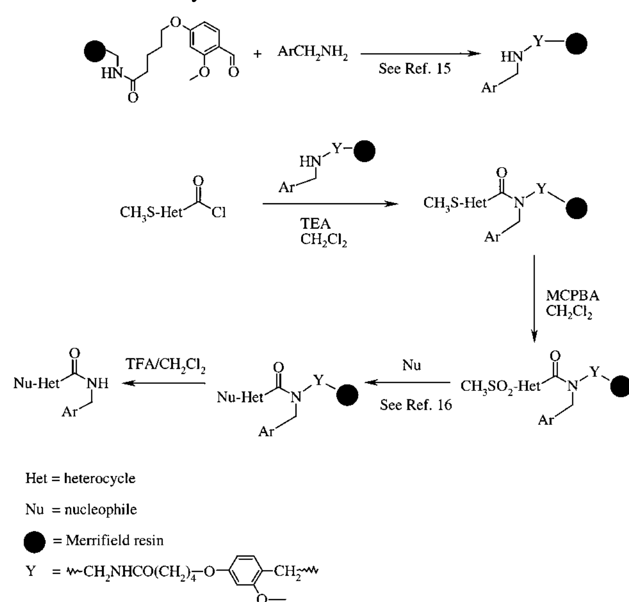
Scheme 1. Library 1



Scheme 2. Library 2



Scheme 3. Library 3



In total, 401 samples belonging to three different structural classes were analyzed. Structure identification was negative or not possible by both HPLC−MS and NMR for 19 of these samples (∼5%). In 47 cases (∼11%) the NMR spectra were too crowded, and therefore, the target compounds were identified by HPLC−MS and only UV purity was measured. In most of the samples (335, 84%) structural confirmation and purity determination were possible by both NMR and HPLC−UV−MS. Purity distributions of 314 samples belonging to this latter set (the remaining 21 did not dissolve completely in DMSO-d_6, and purity values were considered unreliable) are shown in Figure 4, where NMR purity is reported as a percentage of the nominal concentration (concentration that was estimated by the library preparer) otherwise called strength. This latter varies from 5% to 213% with a difference with respect to the estimated concentration ranging from −95% to +133%. An equivalent error would have been introduced in all physicochemical and biochemical data generated with these solutions if concentrations had not been corrected. LC−UV purity distribution is much narrower and cannot be used as a concentration correction factor.

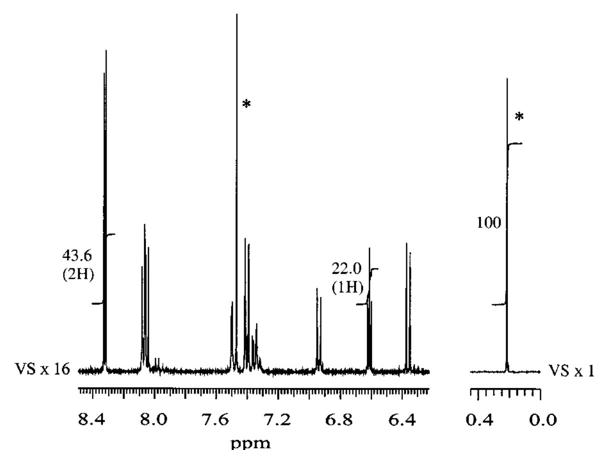


Figure 3. Two portions of the quantitative ^{1}H NMR spectrum of a typical member of library 3. The aromatic and the upfield signals of BSTMS are marked with an asterisk. Vertical scales of the two panels were enhanced with the given factors. The two well-isolated resonances used for strength calculation are shown with the corresponding integral values.

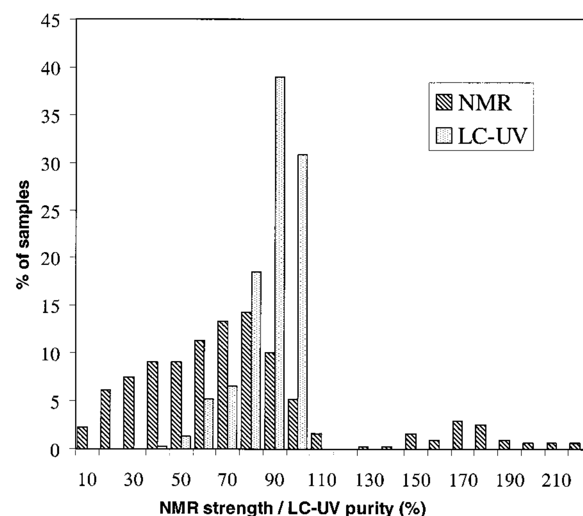


Figure 4. Distribution of NMR strength (percentage of the nominal concentration) for 314 combinatorial chemistry compounds compared with the distribution of the corresponding HPLC−UV purity. Both are reported in 10% steps.

Discussion

After synthesis, a stock DMSO solution of combinatorial chemistry compounds is usually prepared to be used for physicochemical characterization and biological tests. Concentration is estimated on the basis of arbitrary assumptions on reaction and purification yields because these samples are usually not weighable. Even if this were the case, contamination by reagents and reaction byproducts would make concentration obtained in such a way unreliable.

Table 2. In Vitro IC_{50} (μM) of the Four Most Active Combinatorial Compounds in Library 3

compound	IC_{50}, μM	NMR strength, %	corrected IC_{50}, μM	IC_{50} of pure compounds, μM
1	11.3	59	6.7	4.0
2	10.8	63	6.8	7.4
3	20.7	27	5.6	2.1
4	3.0	58	1.7	1.4

A method to measure the absolute concentration (strength) of combinatorial chemistry compounds and therefore to carefully evaluate data obtained with these solutions (especially pharmacological data) is badly needed. For this reason we decided to verify the applicability of our quantitative NMR method to small combinatorial chemistry libraries (100−200 compounds).

The benefit of this effort was apparent when we realized that the estimated concentrations could be largely incorrect, as is clearly shown in Figure 4. The distribution of NMR strengths is much broader than the corresponding LC−UV distribution because this latter is just a relative purity measured upon assuming the sum of all LC−UV peaks equal to 100%. Therefore, either early eluting (solvent front) or column-retained compounds are not considered, and species with a weak (water, salts, solvents, etc.) or strong extinction coefficient are under- or overestimated, respectively. By contrast, NMR takes into account every species contained in the sample with the correct contribution. Of course, the method fails when NMR spectra are overcrowded (∼11% of the samples in our case) and signal overlapping precludes identification and integration of the target molecule resonances. These samples, however, are usually the poorest in purity and should be discarded if the quality of the library has to be maintained at a high level.

A proof of the effectiveness of this approach was afforded by in vitro biological data (shown in Table 2) of the four most active compounds belonging to library 3. Once these molecules were synthesized in pure form to confirm biological activity, IC_{50} values measured on these latter samples were in a good agreement with those generated with combinatorial chemistry solutions after correction of the NMR strength. Notably, the uncorrected IC_{50} of compound **3** is above the threshold for consideration, and this compound would have not been detected as “active”.

Of course, spectral interpretation is mandatory for the application of this method in order to differentiate signals of the target molecule from those of impurities. This is usually not demanding because spectral features of analyzed compounds are well-known from analogues that were prepared during the optimization of the synthetic path. Nevertheless, this is the rate-limiting step of the method (in conjunction with the need for manual data processing), and a tool for automatic signal assignment would speed up the whole process. There is a lot of effort in this area,[9] but as far as we know, a fully reliable software is not yet available.

Experimental Section

Internal Standard. The silane standard BTMSB was obtained from Aldrich at a nominal 96% purity. Purification through sublimation in a coldfinger apparatus at 80 °C and under reduced pressure (∼20 mmHg) afforded a crystalline product of high purity (99.9% according to GC−MS, >99.9% from melting profile analysis) with a melting point of 94.9 °C.

The $^{5}/_{9}$ mM DMSO-d_6 stock solution is prepared by accurately weighing the standard and adding the right amount of solvent with a macro- or micropipet. The suspension is sonicated at 50 °C for 10 min, and after the suspension is regularly shaken, the floating crystals dissolve completely and the solution is allowed to return at room temperature, where it can be stored for at least 30 days.

NMR and MS Samples Preparation. All liquid-handling operations were automatically performed by means of a Tecan Genesis 100 automatic dilutor. A portion of the DMSO-h_6 solution of combinatorial chemistry compounds used for biological tests was concentrated in vacuo and redissolved in the right amount of DMSO-d_6 (purchased from Merck, 99.95% D) to get an estimated 10 mM stock solution. Samples were manually shaken until a clear solution was obtained.

The NMR samples were prepared by mixing 200 μL of the above 10 mM stock solution with 200 μL of the $^{5}/_{9}$ mM BTMSB solution and 200 μL of DMSO-d_6 directly in the NMR tube (Wilmad 507PP). In the final NMR solutions, the concentration ratio of silane to test molecule (assumed 100% pure) is 1:18, which exactly compensates for the 18 protons of the two trimethylsilyl moieties corresponding to the reference signal.

Separate samples were prepared for HPLC−UV−MS. A total of 20 μL of the 10 mM DMSO-d_6 stock solution was diluted to 1.0 mL with a mixture of ammonium acetate buffer (5 mM, pH 5)/acetonitrile, 1:1 (v/v), and a total of 10 μL was injected. DMSO-d_6 is eluted with the solvent front.

In the case of the quantitative NMR validation method, solutions to be assayed were prepared by weighing a few milligrams of each standard compound with a microbalance and adding the right amount of DMSO-d_6 for 10 mM concentration. This solution was mixed with the internal standard and DMSO-d_6, as reported above.

NMR Acquisition Parameter and Data Processing. All NMR spectra were acquired on a Varian MercuryVx-400 instrument (operating at 400.45 MHz for proton) equipped with a 5 mm double resonance $^{1}H\{^{15}N-^{31}P\}$ ID-PFG Varian probe with single axis (z) gradient coil. Samples were loaded into the magnet with an automatic sample changer (Zymark-Zymate XP Robot); deuterium gradient shimming and data acquisition were automatically performed by the acquisition software (VNMR, version 6.1B).

The following are typical quantitative acquisition parameters: sample temperature of 28 °C, preacquisition delay of 2 min to allow temperature equilibration, sample spinning rate of 20 Hz, relaxation delay of 25.0 s, 90° flip angle corresponding to a pulse duration of 6.5 μs, 64 transients (32 transients afford an acceptable S/N for quantitation), and 43 000 complex free induction decay (FID) data points acquired over a spectral width of 8600 Hz (acquisition time 5.0 s). The delay between pulse and acquisition was

optimized (94 ms) for this spectral window in order to have a flat baseline,[17] and therefore, no first-order phase correction was necessary after Fourier transformation.

An acceptable phase and intensity response of the analogue filters (four-pole Butterworth) between −1.0 and 9.0 ppm was obtained using a spectral window of 21.5 ppm. However, the intensity response of the filters was found to be *nonlinear* but reproducible in this range, and it was evaluated using the residual HDO signal in a sample of D_2O. The transmitter offset was arrayed over the whole spectral width in steps of 200 Hz, and the acquisition was repeated three times. Integrals were averaged, and a standardization curve was drawn through interpolation with a sixth-order polynomial that was used as a correction factor $f(\delta)$ for the integrated peak intensity.

To avoid any baseline artifact caused by the transient response of analogue filters (distortion of the first few data points of the FID), the first three FID data points were linearly predicted before Fourier transformation.[17] FIDS were zero-filled to 128K complex data points, and no weighting functions were used. A final S/N greater than 700 measured on the 0.22 ppm internal standard signal was obtained. First-order baseline correction was applied after Fourier transformation and integral regions selection. Chemical shifts were referenced to the residual solvent signal (DMSO-d_5, 2.49 ppm).

For a given molecular species, strength is computed on any assigned peak as follows:

$$\text{strength} = (\text{purity}_{st})(I_{peak})/[n_H f(\delta)\, I_{st}]$$

where $\text{purity}_{st} = 100\%$ and is the assigned purity of the silane standard, n_H is the number of hydrogen atoms associated with the signal, $f(\delta)$ is the analogue filter intensity correction function, I_{peak} is the integrated intensity of the signal peak, and I_{st} is the integrated intensity of silane resonance at 0.22 ppm. Strength is the absolute purity of the solid in the case of weighed samples (quantitative method validation) or the concentration correction factor for combinatorial chemistry solutions. Values obtained from the integration of a few signals are usually averaged to give the final sample strength.

HPLC−UV−MS Method. Mass spectra were recorded on a Finnigan MAT LCQ ion trap instrument equipped with an electrospray (ESI) ion source; the mass spectrometer is directly connected to a Spectra System P4000 HPLC pump (Thermo Separation Products) equipped with an AS3000 autosampler and an UV6000LP diode array detector.

Ions were generated under the following conditions: ESI sprayer voltage of 4.0 kV, heated capillary temperature of 255 °C, and sheat gas nitrogen with a pressure of 5.5 bar. Full-scan spectra in the mass range 100−1000 amu (or 100−2000 amu if necessary) alternated with MS/MS daughter ion scans of the most intense peaks (relative collision energy of the MS/MS scan 35%) and were recorded. Positive and negative ions spectra were acquired in separate chromatographic runs. This double acquisition offers the advantage of collecting more spectra for each type of experiment if compared to alternate positive and negative ions scans on the same chromatographic run.

HPLC chromatography was carried out using a Waters XTerra RP 18 column (3.5 μm particles, 4.6 mm × 50 mm) at room temperature and 1.0 mL/min flow rate. Mobile phase A consisted of 5 mM ammonium acetate−acetic acid buffer (pH 5)/acetonitrile 9:1 (v/v). Mobile phase B consisted of 5 mM ammonium acetate−acetic acid buffer (pH 5)/acetonitrile 1:9 (v/v). Chromatographic separation was achieved using a gradient from 0% to 100% of B in 7 min and isocratic at 100% of B in another 2 min. This was followed by a gradient from 100% to 0% of B in 0.1 min and was isocratic at 100% of A for 0.9 min (total acquisition time was 10 min). The UV detection range was 215−400 nm. For the determination of purity, the strong signal of solvents (DMSO, CH_3CN) were excluded from the integration range.

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CC000101T