Nuclear Spin Singlet Order Selection by Adiabatically Ramped RF-fields

Alexey S. Kiryutin,[a,b] Andrey N. Pravdivtsev, [a,b] Alexandra V. Yurkovskaya, [a,b] Hans-Martin Vieth, [a,c] Konstantin L. Ivanov [a,b] *

[A] *International Tomography Center, Siberian Branch of the Russian Academy of Science, Institutskaya 3A, Novosibirsk, 630090, Russia*

[b] *Novosibirsk State University, Pirogova 2, Novosibirsk, 630090, Russia*

[c] *Institut für Experimentalphysik, Freie Universität Berlin, Arnimallee 14, Berlin, 14195, Germany*

This file contains:

- Filtering efficiency for different spin order
- Results for singlet order selection in other spin systems (i.e., aromatic protons of amino acids, aromatic protons of nucleic acid bases, and ribose protons)
- Performance of the SOS-filter for an ABX-spin system
- Results of SOS-filtering in different residues in Met-enkephalin and ubiquitin
- Discussion of singlet order relaxation due to fluctuating local fields
- Methods for T_2-relaxation measurements utilizing the SOS-filter
- Comparison with other methods for filtering singlet spin order

*** Corresponding author: E-mail: ivanov@tomo.nsc.ru; tel. +7(383)330-8868; fax +7(383)333-1399.**

A. Filtering efficiency for different spin order

We performed additional calculations for revealing, which spin order can pass through the SOS-filter. The density matrix of a two-spin system was the following: $\rho_0 = \frac{1}{4}\hat{E} + \xi\widehat{\mathrm{SO}}$; here ξ is a coefficient, $\widehat{\mathrm{SO}}$ stands for the spin order. We used the following $\widehat{\mathrm{SO}}$:

- Net z-polarization of the two spins, $(\hat{I}_{az} + \hat{I}_{bz})$;
- Difference of z-polarization of the two spins, $(\hat{I}_{az} - \hat{I}_{bz})$;
- Two-spin order $\hat{I}_{az}\hat{I}_{bz}$;
- Zero-quantum order $\widehat{\mathrm{ZQ}} = \hat{I}_{ax}\hat{I}_{bx} + \hat{I}_{ay}\hat{I}_{by}$.

For each spin order we calculated the final density matrix after two RF-switches and evaluated the resulting spin order. The calculation was done with the same parameters as those used in Figure 1. The calculation was performed twice: in the first calculation we assumed that Δ was positive for both RF-switches (RF-on and RF-off); in the second calculation Δ is positive for the RF-on switch and negative for the RF-off switch. Let us briefly summarize the results:

- When the initial spin order is $(\hat{I}_{az} + \hat{I}_{bz})$ after the M2S-S2M conversion of the first kind the final spin order is $(\hat{I}_{az} + \hat{I}_{bz})$; after the M2S-S2M conversion of the second kind the final spin order becomes $-(\hat{I}_{az} + \hat{I}_{bz})$. Spin order of any other kind is negligibly small in both cases. Thus, after subtracting the results of the two experiments we obtain the $(\hat{I}_{az} + \hat{I}_{bz})$ spin order: net polarization passes through the filter.

- When the initial spin order is $\left(\hat{I}_{az} - \hat{I}_{bz}\right)$ after the M2S-S2M conversion of both kinds the final spin order is $\left(\hat{I}_{az} - \hat{I}_{bz}\right)$; any other spin order is negligibly small. Thus, after subtracting the results of the two experiments we obtain no spin order.

- Two-spin order $\hat{I}_{az}\hat{I}_{bz}$ and zero-quantum order $\widehat{\mathrm{ZQ}}$ do not pass through the filter and do not generate any other spin order.

B. Results for singlet order selection in other systems (i.e., aromatic protons of amino acids, aromatic protons of nucleic acid bases, and ribose protons)

By using the APSOC method we generated singlet spin order not only in CH_2-groups, but also in other systems, namely in aromatic amino acids and nucleotides, such as N-acetyl histidine, N-acetyl tyrosine, and cytidine-5′-monophosphate. Additionally, we applied the SOS-filter to get rid of unwanted background signals. These results are shown in **Figures 1SI**, **2SI**, and **3SI**. It is clearly seen that in all cases the aromatic protons of the molecules under study can be selected by the SOS-filter, whereas other NMR signal are strongly suppressed. In N-acetyl histidine the aromatic protons in the H2 and H4 positions represent a weakly-coupled spin pair; thus, the spin dynamics behind spin order conversion is the same as described in the main text (the small coupling of the H4 proton to the β-CH_2 protons can be neglected). In N-acetyl tyrosine the aromatic protons represent two pairs of equivalent spins, the H2-H6 pair and the H3-H5 pair. The coupling between the pairs is relatively weak, therefore, the APSOC method and SOS-filter works in the same way as described in the main text. The example of cytidine-5′-monophosphate shows that it is possible to select also the ribose protons in nucleotides.

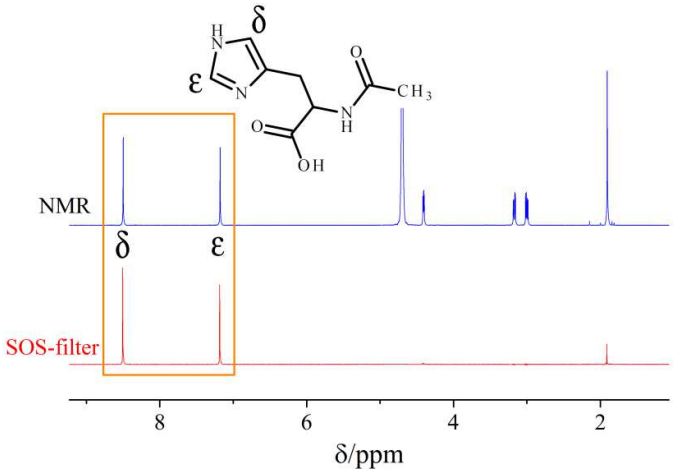

Figure 1SI. Performance of the SOS-filter for the H2 and H4 protons of N-acetyl histidine (30 mM pH 5.5) in D_2O (spin pair belonging to the five-spin system of histidine: α-CH, β-CH_2, δ-CH and ε-CH). SOS-filter parameters are: $\tau_{on} = \tau_{off} = 0.1$ s, $\nu_1^{max} = \nu_{SL} = 4$ kHz, $\nu_0 = 7.842$ ppm, $\Delta = 10$ Hz, $B_0 = 16.4$ T.

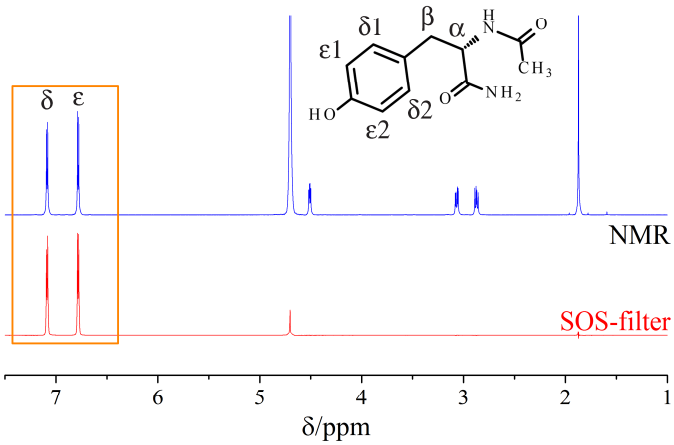

Figure 2SI. Performance of the SOS-filter for the δ-CH_2, and ε-CH_2 protons of N-acetyl tyrosine (30 mM pH 2.5) in D_2O (A_2X_2-spin system). SOS-filter parameters are: $\tau_{on} = \tau_{off} = 0.1$ s, $\nu_1^{max} = \nu_{SL} =$ 1 kHz, $\nu_0 = 6.93$ ppm, $\Delta = 10$ Hz, $B_0 = 16.4$ T.

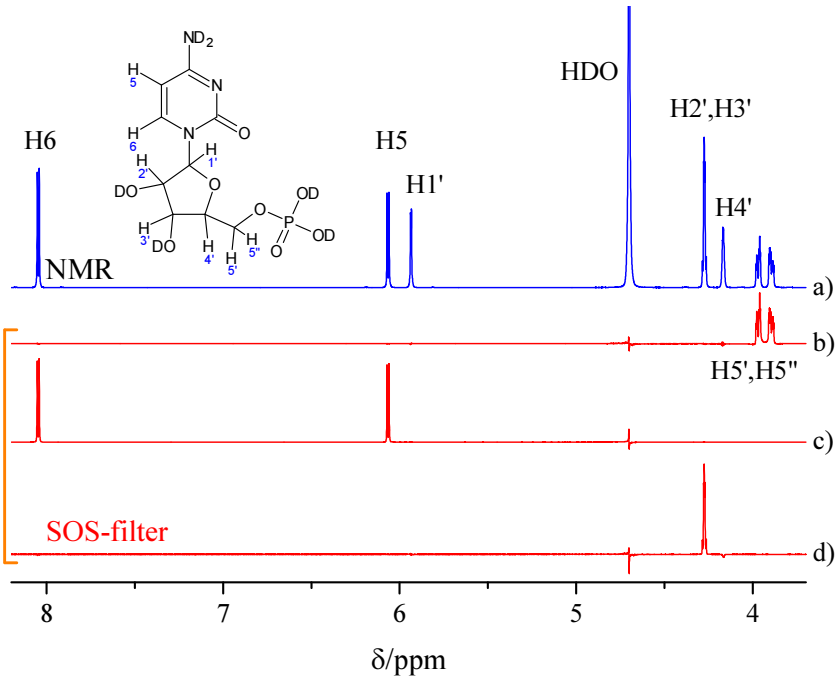

Figure 3SI. Performance of the SOS-filter for the aromatic and ribose protons of cytidine-5′-monophosphate in D_2O: (a) thermal NMR spectrum; (b)-(d) SOS-filtered spectra. SOS-filter parameters are:

(b) $\tau_{on} = \tau_{off} = 0.2$ s, $\nu_1^{max} = \nu_{SL} = 173$ Hz, $\nu_0 = 3.930$ ppm, $\Delta = 10$ Hz, $B_0 = 16.4$ T.

(c) $\tau_{on} = \tau_{off} = 0.1$ s, $\nu_1^{max} = \nu_{SL} = 2.5$ kHz, $\nu_0 = 7.058$ ppm, $\Delta = 6$ Hz, $B_0 = 16.4$ T.

(d) $\tau_{on} = \tau_{off} = 0.2$ s, $\nu_1^{max} = \nu_{SL} = 18$ Hz, $\nu_0 = 4.275$ ppm, $\Delta = 4$ Hz, $B_0 = 16.4$ T.

C. Performance of the SOS-filter for an ABX-spin system

In the main text of the article we always limited the discussion to spin pairs, although, for instance, the β-CH_2 protons in Tyr and Phe are coupled to the neighboring α-CH proton (the coupling strength is equal to approximately 5-7 Hz). In this situation the reduction of the spin system to only a pair of spins is justified because we used a moderate amplitude of the switched RF-fields: ν_1^{max} was not more than 200 Hz, which is much smaller than the difference in the NMR frequencies between the α-CH proton and the β-CH_2 protons. For this reason the α-CH proton is not excited by the RF-field. On the other hand, ν_1^{max} is much stronger than the coupling between the α-CH proton and the β-CH_2 protons; consequently, the RF-field "decouples" the α-CH proton from the spin pair of the β-CH_2 protons. However, when ν_1^{max} is greater than or at least comparable to the difference in the NMR frequencies of the coupled spins the entire three-spin system becomes coupled. Therefore, not the singlet state of the two β-CH_2 protons is created by APSOC, but a three-spin order of the entire system is generated. For this reason, in such a situation NMR signals of all three spins can pass through the SOS-filter, compare **Figure 4SI** (top) with spectrum 3 in Figure 1 in the main text.

The fact that all three spins are affected by APSOC allows us to measure the individual T_1-relaxation times by IR-SOS. Indeed, when all three spins are flipped by a non-selective π-pulse and the delay τ is varied the resulting NMR signals, which pass through the SOS-filter, recover following a bi-exponential time dependence, see **Figure 4SI** (bottom). By fitting this dependence we extract two relaxation times: the fast component has the relaxation time of 0.55 s, which is about the T_1-relaxation time of the β-CH_2 protons; the slow component has a relaxation time of 1.78 s, which is about the T_1-relaxation time of the α-CH_2 protons.

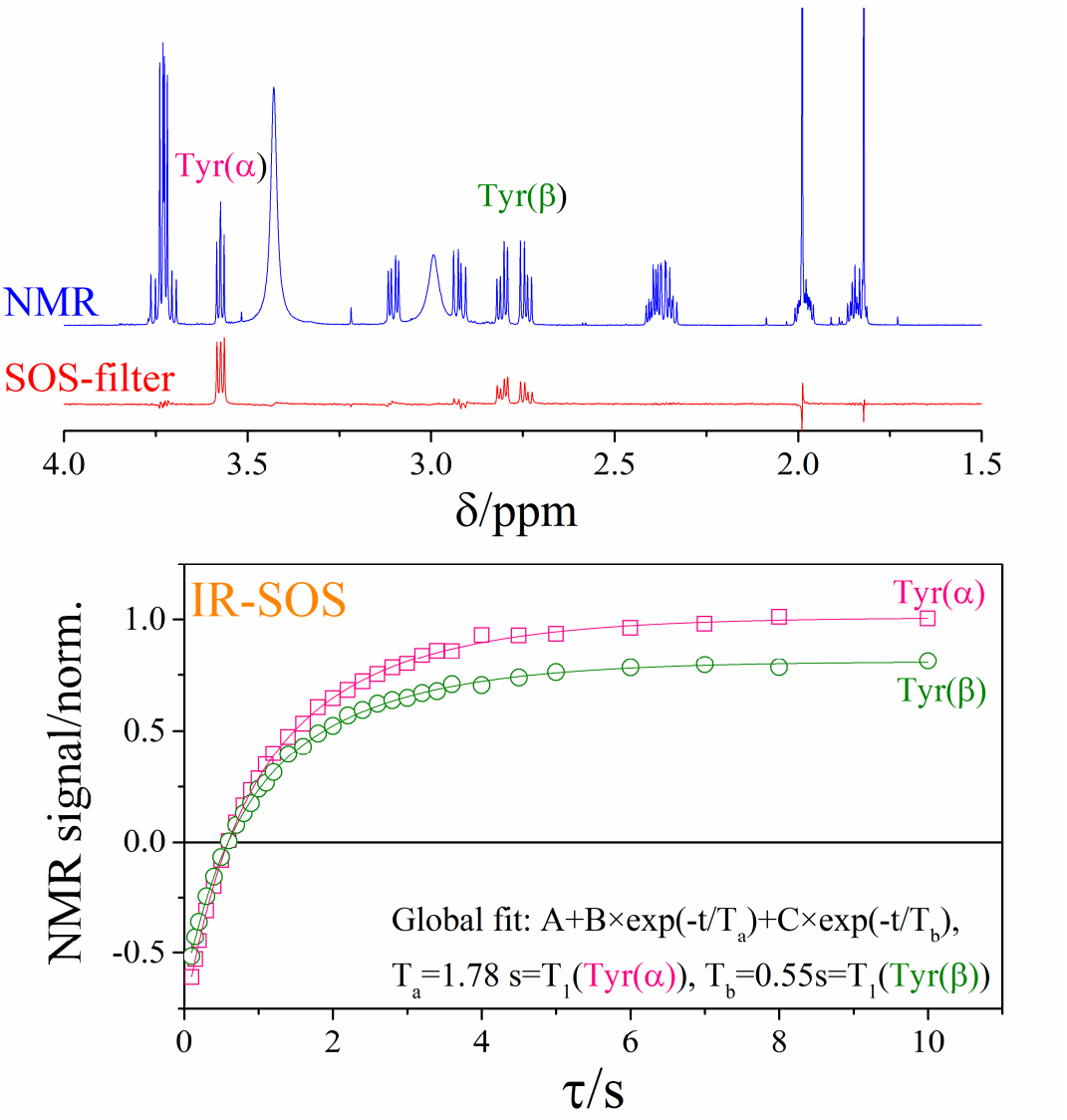

Figure 4SI. (top) Performance of the SOS-filter for an ABX-spin system (α-CH, β-CH_2 protons of Tyr in Met-enkephalin). (bottom) Experimental results for the IR-SOS sequence for the same spin system. The relaxation kinetics can be fitted by a bi-exponential function: the first time constant corresponds to T_1 of Tyr(α) and the second time constant corresponds to T_1 of Tyr(β). SOS-filter parameters are: $\tau_{on} = \tau_{off} = 0.2$ s, $\nu_1^{max} = 2$ kHz, $\nu_{SL} = 4$ kHz, $\tau_{SL} = 0.2$s, $\nu_0 = 3.148$ ppm, $\Delta = 12$ Hz, $B_0 = 16.4$ T.

D. SOS-filtering for different residues in Met-enkephalin and ubiquitin

Enlarged SOS-filtered spectra of different spin pairs in Met-enkephalin are presented in **Figure 5SI**.

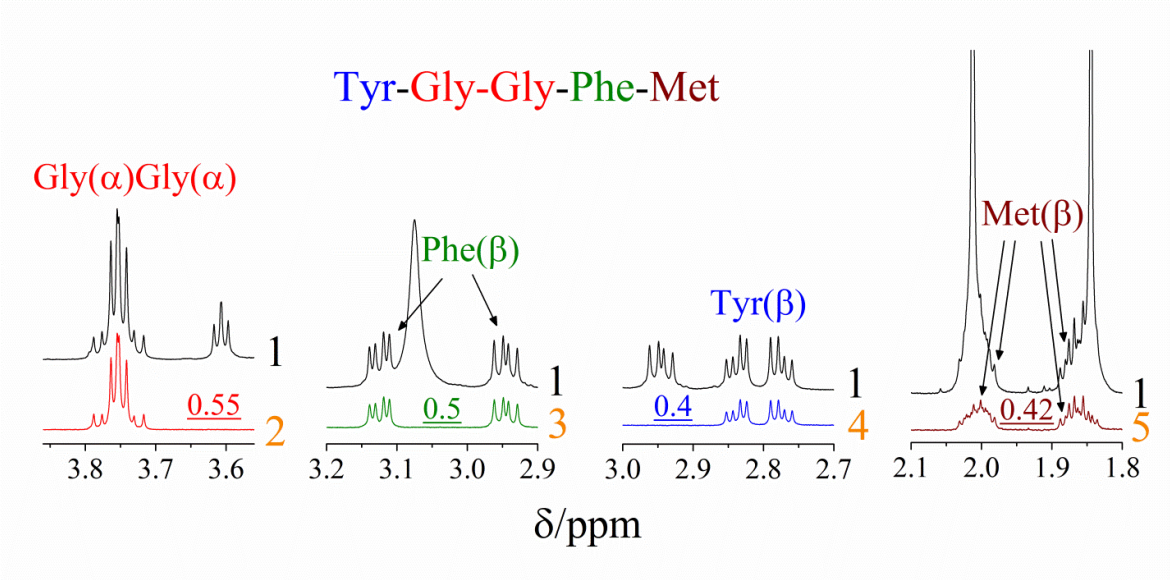

Figure 5SI. ^{1}H-NMR spectra (top, trace 1) and SOS-filtered spectra (bottom) for different spin pairs in Met-enkephalin: two Gly residues (trace 2), Phe (trace 3), Tyr (trace 4), Met (trace 5). Full spectra are shown in Figure 3. Signal intensities, ε, normalized to those in the thermal NMR spectrum are given by underlined number.

Experimental results for the T_1-relaxation times and singlet order lifetimes, T_S, as measured at 16.4 Tesla for the five Gly-residues, are presented in Table 1SI.

Typical time traces for T_1-relaxation (as obtained by IR-SOS experiments) and singlet spin order relaxation (as obtained in SL-SOS experiments) are shown in **Figure 6SI**. The longer T_1 and T_S are the higher is the intensity of the signals passing through the SOS-filter. Notably, for Gly76 having long relaxation times the signal intensity at $\tau_{SL} = 0$ and $\tau = 0$ is above half of the thermal NMR signal. Signal losses at shorter T_1 and T_S are attributed to relaxation during the RF-switches.

These experiments were performed for the following parameters of the SOS-filter:

for Gly76 $\tau_{on} = \tau_{off} = 0.2\ s, \nu_1^{max} = 250\ Hz, \nu_{SL} = 4\ kHz, \tau_{SL} = 0.2\ s, \nu_0 = 3.779$ ppm, $\Delta = 15\ Hz$
for Gly75 $\tau_{on} = \tau_{off} = 0.2\ s, \nu_1^{max} = 125\ Hz, \nu_{SL} = 4\ kHz, \tau_{SL} = 0.2\ s, \nu_0 = 3.970$ ppm, $\Delta = 15\ Hz$

for Gly10 $\tau_{on} = \tau_{off} = 0.2\ s, \nu_1^{max} = 2.8\ kHz, \nu_{SL} = 4\ kHz, \tau_{SL} = 0.2\ s, \nu_0 = 3.976$ ppm, $\Delta = 15\ Hz$
for Gly47 $\tau_{on} = \tau_{off} = 0.2\ s, \nu_1^{max} = 2.6\ kHz, \nu_{SL} = 4\ kHz, \tau_{SL} = 0.2\ s, \nu_0 = 3.774$ ppm, $\Delta = 15\ Hz$
for Gly35 $\tau_{on} = \tau_{off} = 0.2\ s, \nu_1^{max} = 790\ Hz, \nu_{SL} = 4\ kHz, \tau_{SL} = 0.2\ s, \nu_0 = 4.032$ ppm, $\Delta = 15\ Hz$

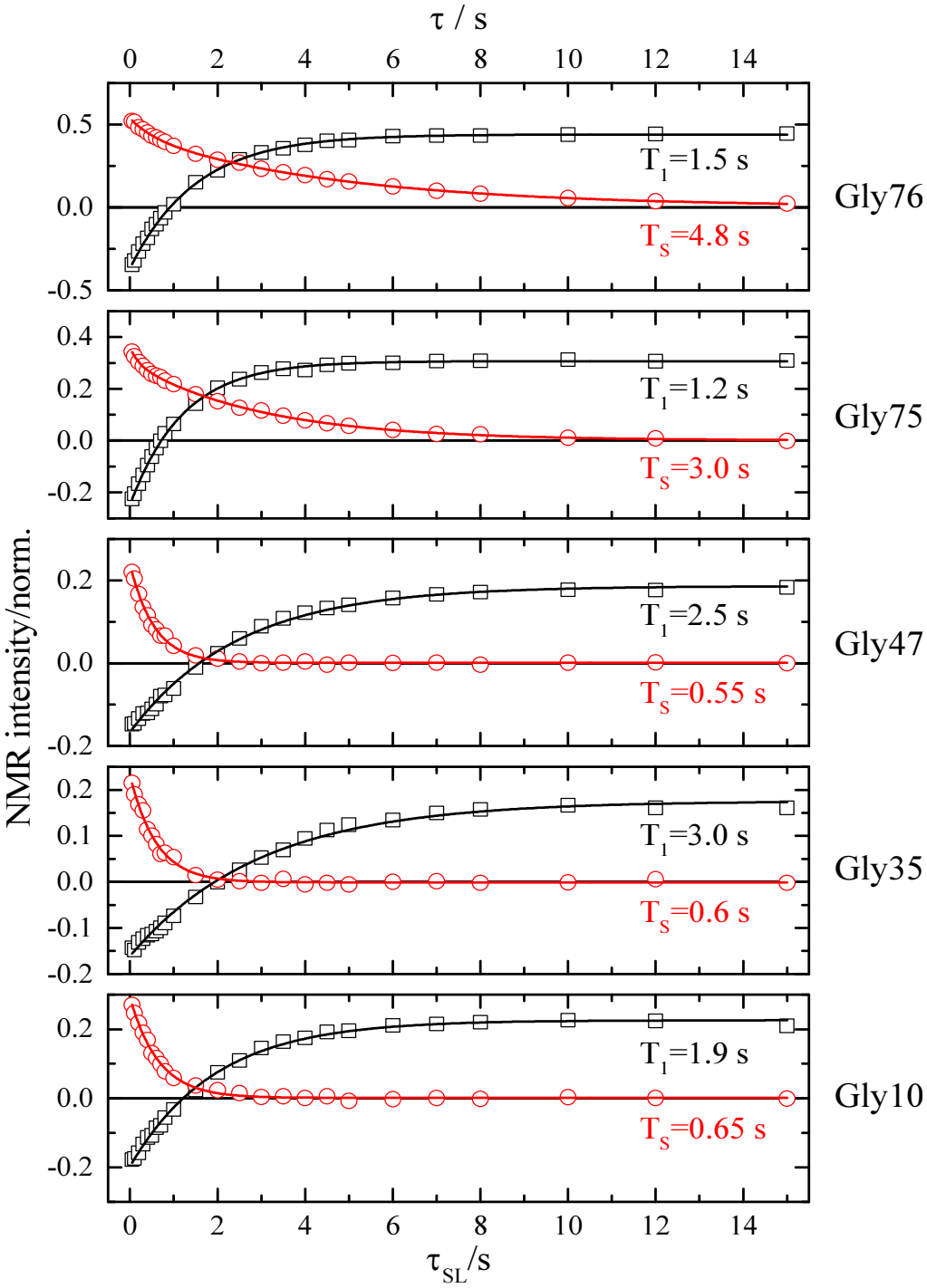

Figure 6SI. Determination of T_1-relaxation times and singlet-order lifetimes, T_S, by using SOS-filter detection for different glycine residues in ubiquitin. The measured NMR intensities are normalized to those in the thermal NMR spectrum. T_1 times are measured using the IR-SOS protocol; T_S times are measured using the SL-SOS protocol.

E. T_S relaxation due to fluctuating local fields

In order to analyze relaxation of the singlet spin order due to interaction with the molecular environment we make use of the results obtained by Freeman et al. [1] and by Carravetta and Levitt [2]. In these works, two models of relaxation were described: (i) relaxation due to fluctuating local fields and (ii) dipolar relaxation. In Ref. [1], the rates of the relaxation transitions between the levels of a two-spin system have been obtained, which are valid for an arbitrary relation between J and $\delta\nu$, but only for fast motions (i.e., the product of the energy gap, ω, as measured in the angular frequency units and the motional correlation time, τ_c, was taken much smaller than one). In Ref. [2] only strongly-coupled spin pairs were considered but the results were generalized to an arbitrary relation between ω and $1/\tau_c$. The expressions derived in Refs. [1,2] are sufficient for our analysis of T_S reduction in proteins.

Here we assume that in proteins dipolar interactions with neighboring nuclei result in local fields, experienced by the spins of the pair under investigation. We denote the spins belonging to this spin pair I_a and I_b and the corresponding local fields as $B_a(t)$ and $B_b(t)$. The local fields fluctuate with time, such that their average values are equal to zero, but their auto-correlation functions exponentially decay to zero with the characteristic time τ_c. Since both spins have a different molecular environment, we assume that $B_a(t)$ and $B_b(t)$ are completely uncorrelated. In this case, assuming that the spin pair is strongly coupled (e.g., by means of a spin-locking RF-field), we obtain that the eigen-states of the spin pair are the singlet state and three triplet states. Then, using the results of Refs. [1,2], we can obtain the following expressions for the longitudinal relaxation rate:

$$R_1 = \frac{1}{T_{1a}} + \frac{1}{T_{1b}} = \frac{2}{3}\gamma^2\langle B_a^2\rangle\mathcal{J}(\omega_N) + \frac{2}{3}\gamma^2\langle B_b^2\rangle\mathcal{J}(\omega_N)$$

Thus, the rate is given by the sum of the two T_1-relaxation rates of the individual spins. Here $\langle B_a^2\rangle$ and $\langle B_b^2\rangle$ are ensemble-averaged values of $B_a^2(t)$ and $B_b^2(t)$; $\mathcal{J}(\omega)$ is the spectral density of

fluctuations at the transition frequency, which is taken equal to the nuclear precession frequency, ω_N, in the external magnetic field. The rates of the transitions, which relax the singlet state, are as follows:

$$R_{S,T_+} = R_{S,T_-} = \frac{1}{4}R_1 = \frac{1}{6}\gamma^2\langle B_a^2\rangle\mathcal{J}(\omega_N) + \frac{1}{6}\gamma^2\langle B_b^2\rangle\mathcal{J}(\omega_N);$$

$$R_{S,T_0} = \frac{1}{6}\gamma^2\langle B_a^2\rangle\mathcal{J}(0) + \frac{1}{6}\gamma^2\langle B_b^2\rangle\mathcal{J}(0)$$

Here we have taken into account that for the $S \leftrightarrow T_\pm$ transitions the energy gap is given by ω_N, whereas for the $S \leftrightarrow T_0$ transitions it is equal to $J \ll 1/\tau_c$. Thus, there are two contributions, which relax the singlet spin order: one of them behaves similarly to T_1-relaxation (as the spectral density at the ω_N frequency comes into play), whereas the other one behaves similarly to T_2-relaxation (as the spectral density at zero frequency comes into play). In proteins, in contrast to small molecules, T_2-relaxation can be much faster than T_1-relaxation: in big molecules at magnetic fields used in modern NMR spectrometers we usually have $\omega_N\tau_c > 1$ (or even $\omega_N\tau_c \gg 1$), consequently, $\mathcal{J}(0) > J(\omega_N)$. Accordingly, in such a situation the T_1-relaxation time can indeed be longer than both T_2 and T_S. At the same time, the T_S relaxation time is expected to be longer than T_2, which is indeed the case in our experiments.

The actual relation between T_1 and T_S depends on the relative contributions of the in-pair dipolar relaxation (which does not lead to singlet-triplet transitions but leads to T_1-relaxation) and the local fields (which are expected to have a stronger effect on T_S rather than on T_1). In the peptide under study the dipolar contribution seems to be stronger, whereas in the protein under study the results are different for different residues: for the flexible Gly75 and Gly76 residues the interactions with the protein environment are weaker than the in-pair dipolar coupling, whereas in the other residues the fluctuating environment gives a stronger contribution to relaxation, which is detrimental for the singlet spin order.

F. Methods for T_2-relaxation measurements utilizing the SOS-filter

In order to measure transverse relaxation times we generated a spin echo signal, i.e., we created transverse x-magnetization by a $\left(\frac{\pi}{2}\right)_y$-pulse, refocused it at $t = \tau$ and converted it back into longitudinal magnetization by an additional $\left(\frac{\pi}{2}\right)_{-y}$-pulse. After that the SOS-filter was applied in the same way as in all other cases, see **Figure 7SI**. The experiment was repeated for different τ-values in order to obtain the relaxation time traces, which were fitted by mono-exponentials to determine T_2 for the pair under study. Using spin echo is a prerequisite for getting rid of inhomogeneous broadening of the NMR signals.

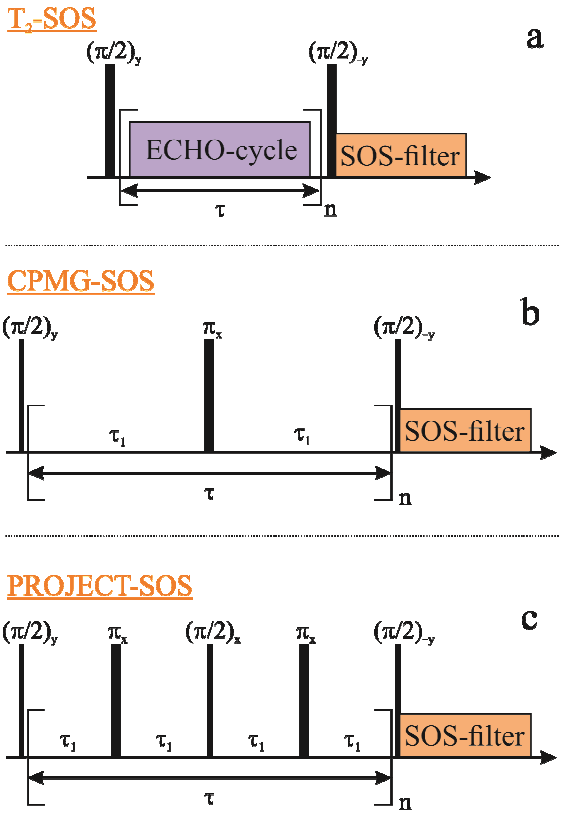

Figure 7SI. General T_2-SOS experimental protocol (a) and pulse sequences used to generate the spin echo: CPMG-SOS (b) and PROJECT-SOS (c).

In experiments we used two schemes for generating the spin echo. The first scheme makes use of the conventional CPMG sequence. However, it is know that in coupled spin systems the spin echo signal is modulated due to spin-spin coupling. This modulation often makes the analysis

more complex, but it can be suppressed by using special pulse sequences, for instance, the so-called PROJECT sequence [3]. Here we used both schemes; due to suppression of the unwanted modulations PROJECT-SOS is more reliable and yields slower decay of magnetization than CPMG-SOS. Typical τ-dependences for T_2-relaxation are given in **Figure 8SI**.

The results of the T_1, T_2 and T_S measurements in ubiquitin are summarized in **Table 1SI**. We have also found that the intensity of the signals passing through the SOS-filter correlates with the measured T_2 values, see **Figure 9SI**. This is an indication that the loss of spin order comes predominantly from T_2-relaxation during the RF-field switches.

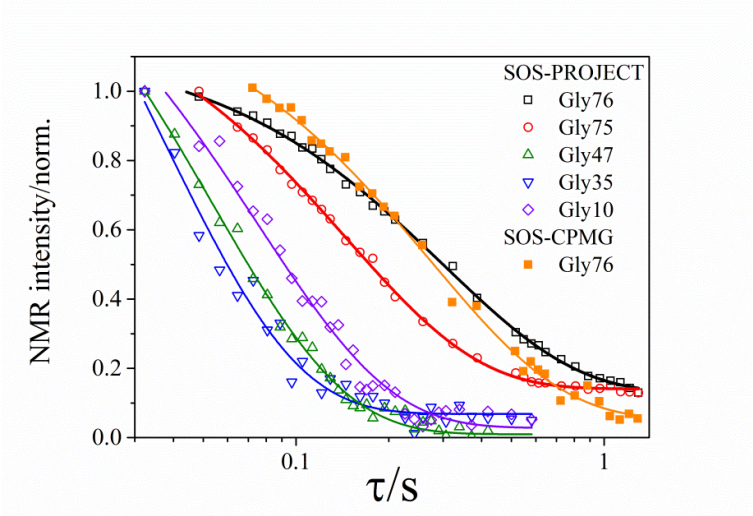

Figure 8SI. Measured τ-dependences for different Gly-residues in ubiquitin measured by PROJECT-SOS; for Gly76 CPMG-SOS was also used. Here $\tau = n \times (4\tau_1 + 5\tau_{90^\circ})$ for PROJECT-SOS and $\tau = n \times (2\tau_1 + 2\tau_{90^\circ})$ for CPMG-SOS. The length of the $\frac{\pi}{2}$-pulses was 7.4 μs, $\tau_1 = 1$ ms.

Table 1SI. T_1, T_2 and T_S values for the α-CH_2 protons of all Gly residues of ubiquitin at 16.4 Tesla (700 MHz), as measured in D_2O.

residue	T_1/s	T_2(SOS-PROJECT)/s	T_S/s	ε
Gly75(α)	1.22	0.145	3.1	0.45
Gly76(α)	1.52	0.30	4.8	0.57
Gly47(α)	2.45	0.052	0.56	0.23
Gly35(α)	3	0.037	0.6	0.23
Gly10(α)	1.95	0.075	0.66	0.31

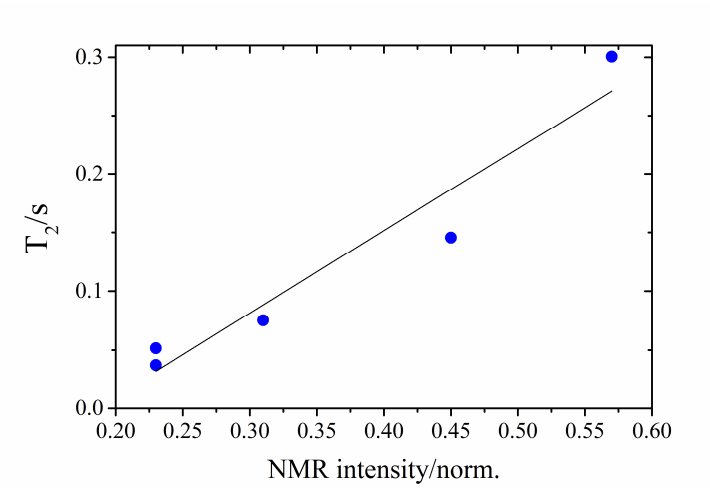

Figure 9SI. Correlation between the intensity of the signals passing through the SOS-filter and the measured T_2 values of the Gly-residues in ubiquitin. Here $\tau_{fs} = 0.2s$ for all measurements; other parameters of the SOS-filter are the same as for Figure 3 of the main text.

G. Comparison with other methods for filtering singlet spin order

We performed filtering of the singlet spin order not only by using the SOS-filter, but also by two methods proposed by Sarkar et al. [4]. These sequences were named sequence II and sequence IV in that paper.

The results are presented in **Figures 10SI** and **11SI**.

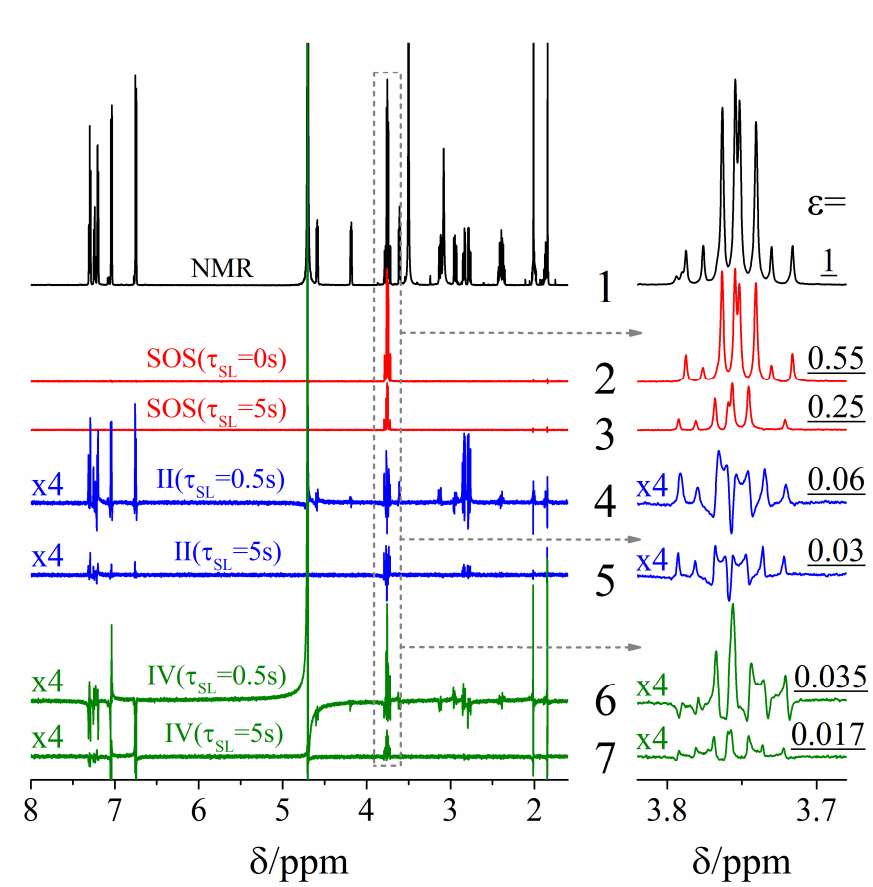

Figure 10SI. 700 MHz ^{1}H NMR spectra of Met-Enkephalin in D_2O. Trace 1 shows the thermal NMR spectrum; traces 2,3 are spectra obtained by the SOS-filter; traces 4,5 are spectra obtained using sequence II from the paper by Sarkar et al. [4]; traces 6,7 are spectra obtained using sequence IV from the paper by Sarkar et al. [4]. In the left we show the full spectral range, while in the right only the signals of the two Gly residues are shown. With each filtering method we acquired two spectra with two different singlet maintenance times. Signal intensities, ε, normalized to those in the thermal NMR spectrum are given by underlined numbers. Experimental parameters are given in the text; all spectra are recorded with 32 accumulations and 8 dummy-scans.

Spectra were taken using the following parameters:

SOS-filter: $\tau_{on} = \tau_{off} = 0.2$ s, $\Delta = 12$ Hz for, $\nu_1^{max} = 44$ Hz, $\nu_0 = 3.753$ ppm, time period of spin-lock is $\tau_{SL} = 0$ s (trace 2) and 5 s (trace 3).

Sequence II of Ref. [4]: $\tau_1 = \frac{1}{4 \cdot 17\text{Hz}}$ and $\tau_2 = \frac{1}{2 \cdot 15\text{Hz}}$, $\tau_{SL} = 0.5$ s (trace 4) and 5 s (trace 5).

Sequence IV of Ref. [4]: $\tau_1 = \frac{1}{4 \cdot 17\text{Hz}}$, $\tau_{SL} = 0.5$ s (trace 6) and 5 s (trace 7). Here we applied the Thrippleton-Keeler filter only before the spin-lock period because we have a probe available only with the z-gradient. After the spin-locking period we waited the same period of time that was used for Thrippleton-Keeler filter before spin-locking but without any manipulations. In this sequence frequency-swept pulses and field gradients are applied simultaneously for suppressing "zero-quantum terms". We used a frequency sweep over approximately 100 kHz; its maximal amplitude was 4.7 kHz and its duration was 10 μs. The field gradient was about 80 kHz.

The WALTZ16 sequence was used for spin-locking.

One can see that all sequences enable the desired suppression of residual NMR lines. The best suppression is achieved with the SOS-filter; among the other two methods sequence IV works better. Both methods proposed by Sarkar et al. [4] improve when the singlet maintenance time is increased, whereas the SOS-filter works equally well for arbitrary spin-locking time. Another advantage of the SOS-filter is that the line shapes are the same as in the thermal spectrum, whereas the other two methods result in distortion of the NMR spectral patterns. On the other hand, a disadvantage of the SOS-filter is that it is not a suitable method for broadband excitation of singlet spin order.

We also checked that in all methods we look at exactly the same spin order. To make such a check we measured the signal intensity as a function of the spin-locking period, see **Figure 11SI**. In all cases, the decay curves of the NMR signal intensity follows a bi-exponential law with the

long relaxation time corresponding to the singlet order lifetime, T_S (which is the same for all methods and equal to 5 s).

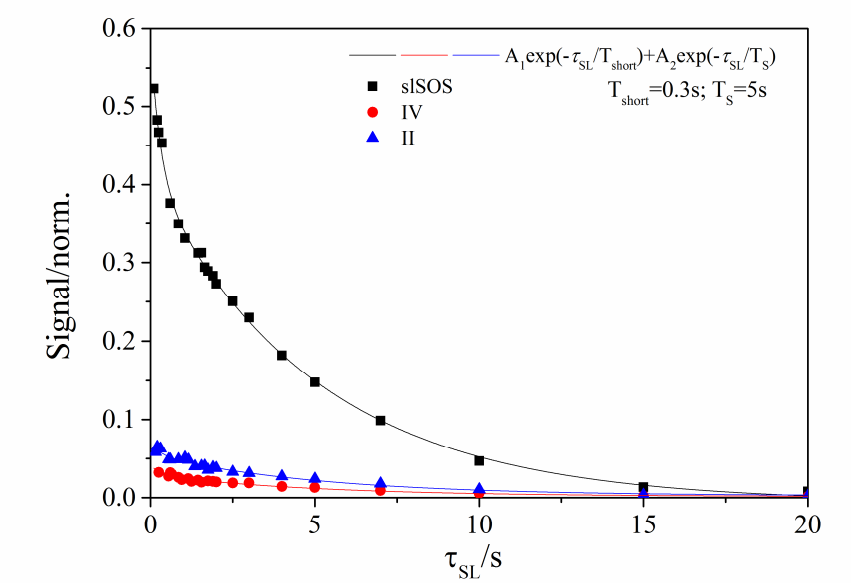

Figure 11SI. Determination of singlet-order lifetime, T_S, by using the SOS-filter (black squares), sequence II of Ref. [4] (blue triangles) and sequence IV of Ref. [4] (red circles). The parameters are the same as in **Figure 9SI**. The decaying curves were fitted by bi-exponential functions (fits are shown by solid lines); in all cases the short decay time is $T_{short} = 0.3$ s and the lifetime of the singlet spin order is $T_S = 5$ s.

References

[1] Freeman, R.; Wittekoek, S.; Ernst, R. R. High-Resolution NMR Study of Relaxation Mechanisms in a Two-Spin System. *J. Chem. Phys*., **1970**, *52*, 1529-1544.

[2] Carravetta, M.; Levitt, M. H. Theory of Long-Lived Nuclear Spin States in Solution Nuclear Magnetic Resonance. I. Singlet States in Low Magnetic Field. *J. Chem. Phys*., **2005**, *122*, 214505.

[3] Segawa, T. F.; Bodenhausen, G. Determination of Transverse Relaxation Rates in Systems with Scalar-Coupled Spins: The Role of Antiphase Coherences. *J. Magn. Reson*. **2013**, *237*, 139-146.

[4] Sarkar, R.; Vasos, P. R.; Bodenhausen, G. Singlet-State Exchange NMR Spectroscopy for the Study of Very Slow Dynamic Processes. *J. Am. Chem. Soc*. **2007**, *129*, 328-334.