ChemComm

Cite this: *Chem. Commun.*, 2011, **47**, 3290–3292

www.rsc.org/chemcomm

COMMUNICATION

Intrinsic pK_a values of 3′-*N*-α-L-aminoacyl-3′-aminodeoxyadenosines determined by pH dependent ^{1}H NMR in H_2O†

Kollappillil S. Krishnakumar,[a] Benoît Y. Michel,[a] Nhat Quang Nguyen-Trung,[a] Bernard Fenet[b] and Peter Strazewski*[a]

Received 23rd November 2010, Accepted 17th January 2011
DOI: 10.1039/c0cc05136e

3′-(α-L-Aminoacylamido)deoxyadenosines are ribosomal A-site binders and mimic the nascent peptide accepting 3′-terminus of aminoacyl transfer RNA. Their α-amino groups exhibit intrinsic basicities in bulk water that differ by up to 1.8 pK_a units. Only the neutral form of these nucleophiles can be active during ribosomal peptidyl transfer catalysis.

The ribosome catalyzes the transfer of nascent peptide chains from 3′-O-esters of P-site bound tRNAs to α-amino groups of A-site bound 3′-aminoacyl-tRNAs. These nucleophilic α-amino groups are shown here to vary considerably in basicity and thus in propensity to be in their neutral reactive form.

In *Escherichia coli* one codon nucleotide triplet is translated into a new peptide bond during an average elongation time of 45 ms at 37 °C.[1] This corresponds to an average protein elongation rate of 22 amino acids per second and ribosome. High *in vitro* elongation rates[1b] are a prerequisite for peptidyl transfer (PT), rather than foregoing aminoacyl-tRNA binding or accommodation steps,[2] to be kinetically observable. Much effort has been spent in the past decade in studies of the molecular details of ribosomal PT through modifications in the peptide-donating P site and its substrates.[3] Peptide-accepting A-site substrates have also been studied. Here knowledge of the basicity and nucleophilicity of the α-amino group of incoming cognate 3′-aminoacyl tRNA (aa-tRNA) is of decisive importance.[4] *In vitro* translation assays, in which aa-tRNA or puromycin (**1**) were used as nascent peptide acceptors, have been carried out at different temperatures and thus elucidated a range of free energies of activation for the ribosome catalyzed PT reaction: $\Delta G_{cat}^{\neq}$ (25 °C) 16.5–12.6 kcal mol^{-1}.[1b,5] Carefully thought-out aqueous model reactions for the bimolecular 'uncatalyzed' ester aminolysis revealed $\Delta G_{uncat}^{\neq}$ (25 °C) 22.2–23.5 kcal mol^{-1}.[5a,6] The difference translates into 10^7 to 10^{10}-fold reaction rate accelerations by the ribosome. Nearly absent or weakly positive PT activation entropies contrast a negative non-enzymatic activation entropy and validate a model according to which the ribosome exhibits throughout the catalyzed reaction pathway firm control over the hydration of the reaction centres and overall H-bonding.[7]

A crucial prerequisite for this understanding is the certainty about the rate-determining step during ribosomal peptide chain elongation at speeds and under conditions that are relevant *in vivo*. For decades one has tried to identify the rate-limiting step in the peptide elongation cycle: after a suggestion that mRNA translocation might be the slowest step followed evidence that A-site occupation prior to PT could be rate limiting. Fluorescence relaxation data have been interpreted to imply that substrate accommodation into the A site is about equally slow for all cognate aa-tRNA and largely pH-independent, and that this step generally determines the overall peptide elongation rate.[8] Conversely, the relative populations of reactive neutral *versus* inactive protonated nucleophiles (α-amino groups of aa-tRNA) are expected to vary quite markedly at physiological pH, depending on the intrinsic pK_a of each nucleophile and on the ability of the ribosomal A-site to shift these intrinsic basicities into a perhaps more favorable apparent regime. Thus, if the chemistry of the PT were rate-limiting, rather than the accommodation of aa-tRNA in the A site, one should expect the rate of protein synthesis to be manifestly dependent on pH close to the physiological range 6–8 and reliant on the pK_a-modulating effect of the 20 different amino acid side chains linked to their A-site bound tRNAs.

Very recent pH dependent ribosomal PT experiments using a fast *in vitro* translation assay unequivocally showed the chemical step to be dominating the transfer rate of formyl methionine from P-site bound fMet-tRNA onto A-site bound prolyl- and glycyl-tRNA at or below physiological pH.[9] MD simulations on atomic coordinates of the large ribosomal subunit containing a P-site bound dipeptidyl-tRNA and each of the experimentally tested Asn-, Phe-, Ile-, Ala-, Gly- and Pro-tRNA were carried out to calculate the pK_a shifts of their α-amino groups as they are being bound into the A-site in the reactant state. For comparison, the experimental pK_a shifts were estimated from the differences between the pK_a values at 25 °C of the α-amino groups of the corresponding natural amino acids, taken from the literature[10a] and downshifted by

[a] *Université Lyon 1, CNRS UMR 5246, Institut de Chimie et Biochimie Moléculaires et Supramoléculaires, Laboratoire de Synthèse de Biomolécules, 43 boulevard du 11 novembre 1918, 69622 Villeurbanne, France. E-mail: strazewski@univ-lyon1.fr*
[b] *CCRMN, Université Lyon 1, CPE-Lyon, Bât. Curien, 43 bd du 11 novembre 1918, F-69622 Villeurbanne, France. E-mail: bfenet@univ-lyon1.fr*

† Electronic supplementary information (ESI) available: Synthetic procedures, NMR data collection, fitting equations and parameters, tables, literature data, SPARC analyses, fitted pH profiles, NMR spectra for titration and compound characterisation. See DOI: 10.1039/c0cc05136e

2.0 pK_a units, and the apparent pK_a values, defined as the pH at which the rate of PT was half maximal at 20 °C.[9] The remarkable correlation suggests that under physiological conditions PT 'chemistry' could, in fact, be rate-limiting for all aa-tRNAs.

The lack of experimental intrinsic (bulk water) pK_a values for aa-tRNA is owed to the hydrolytic instability of the adenosine-3′-O-ester linkage making it impossible to obtain reliable pK_a data for 3′-aminoacyl adenosine esters. Even though α-amino acids and corrections based on experimental pK_a data of simple α-aminoacid methyl or ethyl esters[11] may indeed be good enough models to convey intrinsic pK_a values for α-amino groups of the natural A-site substrates (aa-tRNA), we felt that the current efforts to ultimately understand the ribosomal catalysis of cellular protein synthesis do call for the best possible reference system. We thus decided to experimentally determine pK_a values for the α-amino groups of true nascent peptide acceptors (A-site binders), *viz*. analogues of puromycin that contain various natural or unnatural L-amino acid side chains. In addition, we wished to put into context our data with all available comparable experimental pK_a data of α-amino groups, and with those being estimated by means of a state-of-the-art online calculator named SPARC.[12]

^{13}C and ^{1}H NMR spectroscopic analyses of aminodeoxynucleosides gave pK_a 6.2 and 6.9 for 2′-NH_2 and 3′-NH_2, respectively.[13a,b,d] Most notably, pK_a 6.2 was obtained for 2′-NH_2 in the absence or presence of a vicinal 3′-phosphate group (in dinucleotides). The α-amino group of puromycin, being the most frequently used nascent peptide acceptor analogue, was in the past first merely reported,[13c] then determined from a meticulous 500 MHz ^{1}H NMR spectroscopic study (5 mM in phosphate-buffered D_2O), to exhibit pK_a 7.3 at 25 °C.[13d] Later puromycin was titrated potentiometrically to confer pK_a 6.9 at 37 °C[4a] (*cf*. Table S3, ESI†) and pK_a 7.2 at 25 °C[4d] (both 10 mM initial conc. in H_2O). Analogues of puromycin[4d,f,g] and other α-aminoacid amides[14] were potentiometrically titrated in aqueous solutions that contained no,[14a] small[14b] or ≥ 50% amounts of organic cosolvents. An advantage of ^{1}H NMR spectroscopy over potentiometric pH titrations is the usually lower minimal amount and concentration of the analyte. Puromycin self-associates at ≥ 5 mM in water.[13e] Both π-stacking and organic cosolvents are likely to disfavor ionization and may diminish the apparent pK_a value of the α-amino group. NMR data taken in H_2O/D_2O ≥ 9 : 1, not pure D_2O, produce pK_a values that are comparable without pD–pH correction[15] to pK_a values that have been derived from kinetic assays on ribosomes in H_2O at pH 6–8.[9]

Given the importance of understanding the effect of all twenty incoming natural peptide acceptors on PT catalysis, and most likely on the overall rate of ribosomal protein synthesis, we decided to investigate the full range of intrinsic α-amino group basicities that a ribosomal A site usually has to cope with. The least basic α-amino group of all proteinogenic amino acids is that of L-asparagine (as in **2**, Fig. 1); the most basic α-amino group is that of L-proline (as in **3**). We wondered how strong the pK_a-diminishing effect on an L-proline derivative would be in which a γ-H-atom of the side chain had been replaced by a virtually isosteric F-atom (as in **4**), and what the precise pK_a values are in the glycine- and alanine analogues **5** and **6**, respectively. Here we report on pK_a values of the α-amino group of **1–6** in water. Analogues **7** and **8** served as controls to unmistakably distinguish the effect of α-NH_2 protonation from OH or CONH deprotonation.

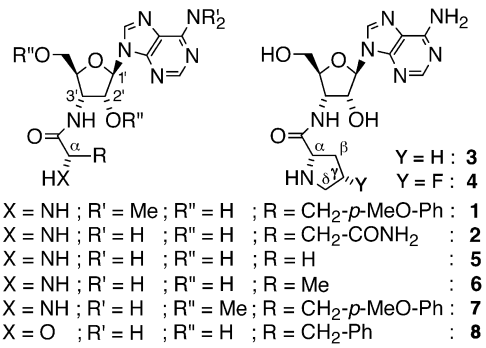

Fig. 1 Puromycin analogues **1–8**.

Asn-, FPro-, Gly-, Ala-, Pro- and L-phenyllactic amides **2–6** and **8** are new puromycin analogues that were synthesized according to published procedures[16] and an optimised protocol for the purification and isolation of the very polar target compounds (ESI†). **7** was obtained from 3′-azido-3′-deoxyadenosine[16] and Me_2SO_4 in aqueous KOH,[17] followed by standard reduction, coupling, deprotection and separation protocols.†‡

For the titration experiments, we avoided any buffer that may interfere with the protonation of α-amino groups, be it through solute complexation or self-dissociation and drastic variation in ionic strength at pH 6–8.[13d] HCl, NaOH, and each analyte at an initial concentration of 1.5 mM were dissolved in H_2O/D_2O 9 : 1–95 : 5 that contained 0.15 M $NaNO_3$, to optimally buffer ionic strength[18] being kept close to physiological values throughout the titration: I = 0.15–0.18 M. All pH-dependent ^{1}H NMR spectra were taken at thermostated 298 K and 600 MHz (**1** and **7**)‡ or 500 MHz (**2–6** and **8**).§ The pH profiles of the amino acid side chain and anomeric ^{1}H NMR signals of puromycin (**1**) and analogues **2–8** showed that signal shifts at pH 5.5–10 are absent only in **8**, are thus due to the ionization of α-NH_2 (Fig. S3, Table S2, ESI†). H1′ signal shifts at pH < 4.5 (in **1–8**) and pH > 11 (in all but **7**) attest ionizations of adenine and, respectively, OH not CONH (Fig. S2, Table S1, ESI†).

In the following list, the results are summarized in columns **A** (compound no. and side chain) and **B** (experimental mean of intrinsic pK_a values at 25 °C for the α-amino groups ± 0.01–0.07, *cf*. Table S2, ESI†). They are compared to the predicted pK_a values of the corresponding 3′-deoxyadenosine-3′-α-aminoacyl amides (**C**: same compounds as in **A**) and the corresponding 3′-esters (**D**), as calculated by SPARC v4.5 for 25 °C (ibmlc2.chem.uga.edu/sparc). Experimental pK_a values from potentiometric titrations at 25 °C of simple α-aminoacyl amides (in 0.15 M NaCl)[14a] and methyl esters (in 0.1 M KCl)[11a] are shown[10b] in columns **E** and **F**, respectively. They demonstrate that the replacement of α-$CONH_2$ with α-$COOCH_3$ reduces the α-amino group's basicity by 0.18–0.45 pK_a units (0.48 and 0.51 for Tyr and Val). The median difference is 0.31 pK_a units (*cf*. Table S3, ESI†) and reflects the electronegativity difference for 3′-O *vs*. 3′-N.

	A	B	C	D	E	F	G	H
2	(Asn)	6.76	6.19	6.07			6.55–6.60	5.9
4	(FPro)	6.77	6.65	6.58			6.56–6.61	
	(Phe)		7.10	6.91	7.45/7.22	7.0		6.1
1	(MeTyr)	7.49	7.47	7.28			7.28–7.33	
	(Ile)		7.50	7.31				6.1
	(Leu)		7.50	7.31	8.00/7.80	7.62		
5	(Gly)	7.95	7.48	7.29	8.03	7.66/7.73	7.74–7.79	7.36
6	(Ala)	8.16	7.50	7.31	8.02	7.78	7.95–8.00	6.3
3	(Pro)	8.55	8.63	8.56	8.82		8.34–8.39	7.8

For **1–6**, both, pK_a values and differences are somewhat underestimated by SPARC—most pronounced for Asn, Ala and Gly (not Pro) 3′-amides—which predicts a corresponding median amide *vs.* ester difference of 0.19 pK_a units. Column **G** shows the accordingly *derived* (**B** − 0.31) *intrinsic* pK_a values for 3′-esters at 20 °C (same compounds as **D**); cooling by 5 °C means to increase pK_a by 0.10–0.15 units.[9,11] The *apparent* α-amino pK_a values of A-site bound aa-tRNA at 20 °C (±0.04–0.2) are reproduced from ref. 9 in **H**.

The apparent basicities of aa-tRNA's nucleophilic α-amino groups that are reacting in the ribosome's A site can differ by up to 1.9 pK_a units (for Asn- *vs.* Pro-tRNA).[9] Given equimolar total concentrations and a local pH 7.5, the nucleophiles of α-amino-Asn : Pro thus appear neutral in a 3 : 1 molar ratio ($[1 + 10^{7.8-pH}]/[1 + 10^{5.9-pH}]$, **H**) (ESI†). This study shows that, within ≤0.2 pK_a limits, the intrinsic basicities of the same α-amino groups at 20 °C (**G**) compare well with earlier less directly derived values[9] and span about the same 1.8 pK_a (**B**) as that of A site-bound aa-tRNA. Molar ratios for neutral (equimolar total) Asn : Pro α-amines in bulk water at pH 7.5 are 10 : 1 for 3′-amides **2** : **3** (Fig. S1, ESI†) and 7–8 : 1 for 3′-esters. FPro **4** sterically stands in for Pro **3** but its α-NH is as weakly basic as that of Asn **2**.

We thank Måns Ehrenberg and Anthony Foster for helpful discussions. Financial support was provided by FP6 programme *Synthcells* and COST Action CM0703 *Systems Chemistry*.

Notes and references

‡ The synthesis and pH-dependent ^{1}H NMR spectroscopic analysis of **1** and **7** were part of the PhD thesis of N. Q. Nguyen-Trung, 2003, University of Basel, Switzerland.

§ Prior to fitting and plotting, the to be analysed chemical shifts δ_H of all compounds **1–8** were corrected for the pH dependence of the internal standard $(CH_3)_3SiCD_2CD_2COONa$ (TSP): $\delta_H(TSP) \equiv 0.00$ ppm, pK_a (TSP) = 5.00, *via* the Henderson–Hasselbalch equation $\delta_H(pH) = 2.217 + \{-2.217 - 2.236 \cdot 10^{pH-5.00}/(1 + 10^{pH-5.00})\}$.[19] All corrected datapoints $\delta_{H\alpha}$, $\delta_{H\beta}$, $\delta_{H\gamma}$, $\delta_{H\delta}$ furnished pH profiles that are best fits for $\delta_H(pH) = \delta_A + (\delta_B - \delta_A) \cdot 10^{pH-pK_a}/(1 + 10^{pH-pK_a})$, where the base lines ($\delta_A$, δ_B) and the transition midpoint (pK_a) served as free fitting parameters.†

1 (*a*) M. Y. Pavlov, R. E. Watts, Z. Tan, V. W. Cornish, M. Ehrenberg and A. C. Forster, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **106**, 50; (*b*) M. Johansson, E. Bouakaz, M. Lovmar and M. Ehrenberg, *Mol. Cell*, 2008, **30**, 589.

2 S. Ledoux and O. C. Uhlenbeck, *Mol. Cell*, 2008, **31**, 114.

3 (*a*) G. K. Das, D. Bhattacharyya and D. P. Burma, *J. Theor. Biol.*, 1999, **200**, 193; (*b*) S. Dorner, C. Panuschka, W. Schmid and A. Barta, *Nucleic Acids Res.*, 2003, **31**, 6536; (*c*) J. S. Weinger, K. M. Parnell, S. Dorner, R. Green and S. A. Strobel, *Nat. Struct. Mol. Biol.*, 2004, **11**, 1101; (*d*) M. D. Erlacher, K. Lang, N. Shankaran, B. Wotzel, A. Hüttenhofer, R. Micura, A. S. Mankin and N. Polacek, *Nucleic Acids Res.*, 2005, **33**, 1618; (*e*) M. M. Changalov, G. D. Ivanova, M. A. Rangelov, P. Acharya, S. Acharya, N. Minakawa, A. Foeldesi, I. B. Stoineva, V. M. Yomtova, C. D. Roussev, A. Matsuda, J. Chattopadhyaya and D. D. Petkov, *ChemBioChem*, 2005, **6**, 992; (*f*) T. M. Schmeing, K. S. Huang, D. E. Kitchen, S. A. Strobel and T. A. Steitz, *Mol. Cell*, 2005, **20**, 437; (*g*) T. M. Schmeing, K. S. Huang, S. A. Strobel and T. A. Steitz, *Nature*, 2005, **438**, 520; (*h*) M. D. Erlacher, K. Lang, B. Wotzel, R. Rieder, R. Micura and N. Polacek, *J. Am. Chem. Soc.*, 2006, **128**, 4453; (*i*) J. S. Weinger and S. A. Strobel, *Biochemistry*, 2006, **45**, 5939; (*j*) M. V. Rodnina, M. Beringer and W. Wintermeyer, *Trends Biochem. Sci.*, 2007, **32**, 20; (*k*) M. Erlacher, D. N. Wilson, R. Micura and N. Polacek, *Chem. Biol.*, 2008, **15**, 485; (*l*) M. Koch, Y. Huang and M. Sprinzl, *Angew. Chem., Int. Ed.*, 2008, **47**, 7242.

4 (*a*) V. I. Katunin, G. W. Muth, S. A. Strobel, W. Wintermeyer and M. V. Rodnina, *Mol. Cell*, 2002, **10**, 339; (*b*) R. Green and J. R. Lorsch, *Cell*, 2002, **110**, 665; (*c*) A. C. Seila, K. Okuda, S. Núñez, A. F. Seila and S. A. Strobel, *Biochemistry*, 2005, **44**, 4018; (*d*) K. Okuda, A. C. Seila and S. A. Strobel, *Biochemistry*, 2005, **44**, 6675; (*e*) B. Zhang, Z. Tan, L. Gartenmann Dickson, M. N. L. Nalam, V. W. Cornish and A. C. Forster, *J. Am. Chem. Soc.*, 2007, **129**, 11316; (*f*) D. A. Kingery, E. Pfund, R. M. Voorhees, K. Okuda, I. Wohlgemuth, D. E. Kitchen, M. V. Rodnina and S. A. Strobel, *Chem. Biol.*, 2008, **15**, 493; (*g*) K. Okuda, T. Hirota, D. A. Kingery and H. Nagasawa, *J. Org. Chem.*, 2009, **74**, 2609.

5 (*a*) A. Sievers, M. Beringer, M. V. Rodina and R. Wolfenden, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 7897; (*b*) M. Beringer, C. Bruell, L. Xiong, P. Pfister, P. Bieling, V. I. Katunin, A. S. Mankin, E. C. Böttger and M. V. Rodnina, *J. Biol. Chem.*, 2005, **280**, 36065; (*c*) I. Wohlgemuth, S. Brenner, M. Beringer and M. V. Rodnina, *J. Biol. Chem.*, 2008, **283**, 32229.

6 (*a*) A. Sievers, M. Beringer, M. V. Rodina and R. Wolfenden, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 12397; (*b*) G. K. Schroeder and R. Wolfenden, *Biochemistry*, 2007, **46**, 4037.

7 (*a*) P. K. Sharma, Y. Xiang, M. Kato and A. Warshel, *Biochemistry*, 2005, **44**, 11307; (*b*) S. Trobro and J. Åqvist, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 12395; (*c*) S. Trobro and J. Åqvist, *Biochemistry*, 2006, **45**, 7049; (*d*) A. Gindulyte, A. Bashan, I. Agmon, L. Massa, A. Yonath and J. Karle, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 13327; (*e*) G. Wallin and J. Åqvist, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 1888.

8 (*a*) M. V. Rodnina, R. Fricke and W. Wintermeyer, *Biochemistry*, 1994, **33**, 12267; (*b*) T. Pape, W. Wintermeyer and M. V. Rodnina, *EMBO J.*, 1998, **17**, 7490.

9 M. Johansson, K.-W. Ieong, S. Trobro, P. Strazewski, J. Åqvist, M. Y. Pavlov and M. Ehrenberg, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **108**, 79.

10 (*a*) A. Fersht, *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*, Freeman & Co, New York, 1999; (*b*) H. A. Sober, *CRC Handbook of Biochemistry*, 2nd edn, 1970.

11 (*a*) R. W. Hay and L. J. Porter, *J. Chem. Soc. B*, 1967, 1261; (*b*) R. W. Hay and P. J. Morris, *J. Chem. Soc. B*, 1970, 1577.

12 (*a*) S. H. Hilal, S. W. Karickhoff and L. A. Carreira, *Quant. Struct.–Act. Relat.*, 1995, **14**, 348; (*b*) A. C. Lee and G. M. Crippen, *J. Chem. Inf. Model.*, 2009, **49**, 2013; (*c*) D. D. Perrin, B. Dempsey and E. P. Serjeant, *pK$_a$ Prediction for Organic Acids and Bases*, Chapman & Hall, New York, 1981.

13 (*a*) P. S. Miller, B. Purshotam and K. Lou-Sing, *Nucleosides, Nucleotides Nucleic Acids*, 1993, **12**, 785; (*b*) H. Aurup, T. Tuschl, F. Benseler, J. Ludwig and F. Eckstein, *Nucleic Acids Res.*, 1994, **22**, 20; (*c*) G. M. Cathey and S. J. Klebanoff, *Biochim. Biophys. Acta*, 1967, **145**, 806; (*d*) S. S. Narula and M. M. Dhingra, *Indian J. Biochem. Biophys.*, 1986, **23**, 306; (*e*) S. S. Narula and M. M. Dhingra, *J. Biomol. Struct. Dyn.*, 1984, **2**, 191.

14 (*a*) R. W. Chambers and F. H. Carpenter, *J. Am. Chem. Soc.*, 1955, **77**, 1523; (*b*) R. E. Watts and A. C. Forster, *Biochemistry*, 2010, **49**, 2177.

15 A. Krężel and W. Bal, *J. Inorg. Biochem.*, 2004, **98**, 161.

16 (*a*) H. Chapuis and P. Strazewski, *Tetrahedron*, 2006, **62**, 12108; (*b*) A. Charafeddine, W. Dayoub, H. Chapuis and P. Strazewski, *Chem.–Eur. J.*, 2007, **13**, 5566; (*c*) B. Y. Michel, K. S. Krishnakumar and P. Strazewski, *Synlett*, 2008, 246; (*d*) B. Y. Michel and P. Strazewski, *Chem.–Eur. J.*, 2009, **15**, 6244.

17 J. T. Kazimierczuk, E. Darzynkiewicz and D. Shugar, *Biochemistry*, 1976, **15**, 2735.

18 R. Sigel and H. Sigel, *Acc. Chem. Res.*, 2010, **43**, 974.

19 A. De Marco, *J. Magn. Reson.*, 1977, **26**, 527.

