Inhibition of *Escherichia coli* RecA by rationally redesigned N-terminal helix†‡

Daniel J. Cline, Shannon L. Holt and Scott F. Singleton*

Received 1st March 2007, Accepted 10th April 2007
First published as an Advance Article on the web 18th April 2007
DOI: 10.1039/b703159a

Bacterial RecA promotes the development and transmission of antibiotic resistance genes by self-assembling into an ATP-hydrolyzing filamentous homopolymer on single-stranded DNA. We report the design of a 29mer peptide based on the RecA N-terminal domain involved in intermonomer contact that inhibits RecA filament assembly with an IC_{50} of 3 μM.

Drug resistance is an ever-increasing problem for modern chemotherapy of bacterial infectious diseases.[1–3] Although the mechanisms that facilitate the *de novo* development, clonal spread, and horizontal transfer of resistance factors are not fully understood, the rapid rate at which antibiotic-resistant bacteria appear is largely due to mutations arising during stress-induced DNA repair[4–7] and gene transfer between organisms.[8,9] Recently, the bacterial RecA protein has emerged as a crucial player in these phenomena.[5–9] Interestingly, RecA has long been known to influence the ability of bacteria to overcome the metabolic stress induced by a range of antibacterial agents,[8,10–26] and its functions are also important for other aspects of bacterial pathogenicity, including the colonization of host environments,[27] induction of toxin biosynthesis,[17,28] virulence factor production,[14] antigenic variation,[29] and survival responses to antibiotic chemotherapy.[1,10,12,30] These activities, which are ubiquitous among bacterial species, make RecA an attractive target to attenuate the rate at which bacterial pathogens become resistant to antibiotic chemotherapy.

In spite of RecA's remarkably diverse set of biological activities, all but one of the protein's known functions require formation of an active RecA–DNA filament comprising multiple RecA monomers (each bound to a molecule of ATP) stoichiometrically coating DNA to form a multimeric right-handed helical filament with about 6 RecA monomers and 18 DNA base pairs per turn.[31] Hence, the discovery of small molecules that suppress the formation of such RecA–DNA filaments would be an important step in the development of agents that modulate the evolution and transmission of antibiotic resistance genes. Unfortunately, no natural products have been definitely characterized as inhibitors of RecA's activities.[32,33] To overcome this limitation, we have rationally developed metal–dithiol complexes[32] and nucleotide analogues[34,35] that inhibit the *in vitro* activities of pre-formed RecA–DNA filaments. In this report, however, we describe a peptide inhibitor designed to prevent the assembly of RecA–DNA filaments—an obligatory early intermediate in all of RecA activities—by disrupting the monomer–monomer interfacial contact region.

The α-helix A and β-sheet 0 (Fig. 1, blue) of one RecA monomer form the key interfacial contacts with helix E and sheet 4 (orange) of the adjacent monomer in the crystal structure reported by Story *et al.*[36] Previous studies have established the importance of this contact region, where N-terminal truncation mutants were deficient in ATPase activity and filament formation.[37–42] Disruption of interfacial contact regions by peptides has been previously

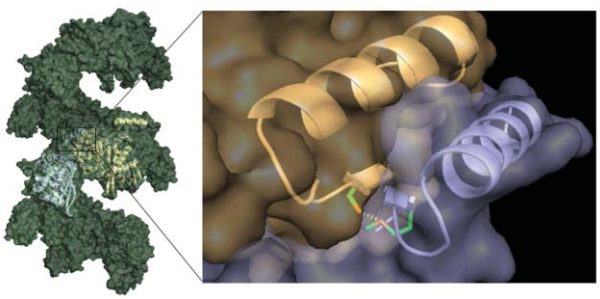

Fig. 1 RecA filament and intermonomer interface. The N-terminal helix (blue) packs against the adjacent monomer (orange). Note Cys116 and Met 27 sidechains are within H-bonding distances (2.8 Å, dashed line).

School of Pharmacy, The University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599-7360, USA. E-mail: sfs@email.unc.edu; Fax: +1 919-966-0204; Tel: +1 919-966-7954

† Electronic supplementary information (ESI) available: CD spectra for peptides, ATPase assays in presence of DTT, MALDI-TOF results for in-gel chymotrypsin digestion of RecA-peptide conjugates, RP-HPLC assessment of peptide purity. See DOI: 10.1039/b703159a

‡ *Materials and Methods* MBHA Rink amide resin, HBTU, HOBt, and appropriately side-chain protected Fmoc-amino acids were purchased from SynPep (Dublin, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), and solvents were from Fisher (Somerville, NJ). HPLC solvent A was 95% water, 5% acetonitrile, 0.1% TFA, and HPLC solvent B was 5% water, 95% acetonitrile, 0.1% TFA.

Peptide synthesis INPEPs were prepared using standard manual Fmoc-solid phase peptide synthesis protocols, cleaved from resin for 3 h using TFA : thioanisole : anisole : triisopropylsilane : water (88 : 5 : 3 : 2 : 2), and purified by reverse-phase HPLC on C-18 column using a linear gradient of 0% to 26% HPLC solvent B in HPLC solvent A over 13 min followed by an additional gradient 26–36% over 20 min. The physical purities of the peptides were estimated to be >98% using analytical HPLC with a linear gradient of 0 to 100% HPLC solvent B in HPLC solvent A over 100 min (see Fig. S4 in ESI). Purified INPEP was identified by ESI-MS: calc'd $(M + H)^+ = 3222.8$, found 3222.3. Pure reduced peptide (INPEP-SH) ESI-MS: calc'd $(M + H)^+ = 3194.8$, found 3195.0. Thiopyridine activated peptide (INPEP-STP) was obtained from INPEP-SH by a modification of a known protocol.[56] Briefly, INPEP-SH (7.0 mg) was dissolved in a 1:3 mixture of HPLC solvents A to B at 0.1 mg mL^{-1} and reacted with 20 eq. 2,2′-dithiodipyridine for 30 min. After removal of the acetonitrile by rotary evaporation, the remaining solution was purified by HPLC (see above) and identified by ESI-MS: calc'd $(M + H)^+ = 3304.8$, found 3304.0. Acetylated peptide (INPEP-SAlk) was obtained from reaction of 0.9 mM INPEP-SH with 1 mM iodoacetamide in 100 mM potassium phosphate, pH 7.2. After 4 h, the product was isolated by HPLC (see above, peak eluted at 23 min) ESI-MS: calc'd $(M + H)^+ = 3252.8$, found 3251.5.

Abbreviations used in text: phosphoenolpyruvate, PEP, lactic dehydrogenase, LDH, pyruvate kinase, PK, dithiothreitol, DTT, 2-(1H-benzotriazole-1-yl)–1,1,3,3-tetramethyluronium hexafluorophosphate, HBTU, *N*-hydroxybenzotriazole, HOBt, trifluoroacetic acid, TFA.

reported as an effective strategy for antagonizing protein–protein interactions (for recent reviews, see references 43–45). With RecA, however, the N-terminal 30 amino acids proved ineffective as an inhibitor of RecA activity, requiring a 12 h pre-incubation at 4 °C to yield IC_{50} of ≥ 500 μM (data not shown). A cursory inspection of the N-30 sequence suggested several elements which could lead to relaxed secondary structure in the 30mer. Therefore, a rational design strategy was employed to increase the secondary structural elements involved in the interfacial contact to improve potency in our designed INhibitory PEPtide (INPEP, Fig. 2).

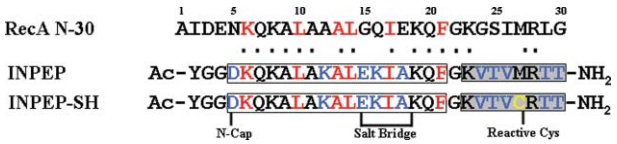

Fig. 2 Design of INPEP based on RecA N-terminal domain.

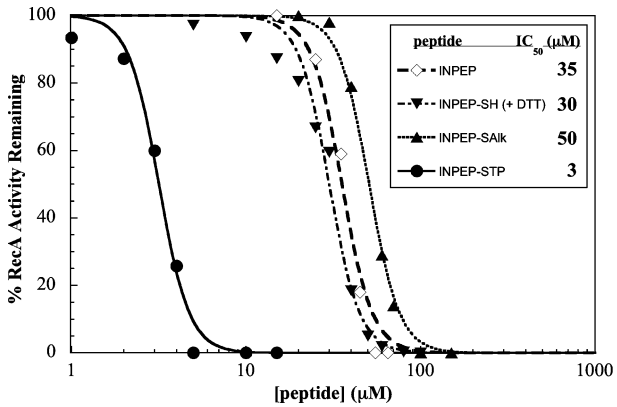

Fig. 3 RecA *in vitro* ATPase activity inhibition. Dose dependent inhibition of RecA ATPase activity by INPEP (◊), INPEP-SH with 2 mM DTT present (▼), unreactive, alkylated thiol INPEP-SAlk (▲), and thiopyridine-activated thiol INPEP-STP (●).

Residues involved in key contacts between monomers (red) were retained on the designed helical peptide, while residues not involved in binding were changed to increase structural stability (blue). The contact region of the native peptide begins at the Asn in position 5. Here, the neutral Asn residue was changed to a charged residue Asp to cap the N-terminal dipole of the α-helix (Fig. 2, boxed sequence), and thus acts as an initiator of helix formation.[46,47] Residues Ala12 and Gln16 were changed to Lys to improve water solubility. Gly15 was changed to Glu to both provide an *i*, *i* + 4 salt bridge with Lys19[48] and improve helicity. Glu18 was changed to an Ala to increase the positive character of the peptide while maintaining the helical propensity at this position. Replacing the anionic Glu residue with Ala was expected to decrease the potential for inter-peptide salt-bridge formation that would promote aggregation, and to increase the Coulombic attraction between the peptide and RecA (pI < 7). Ser25, Leu29, and Gly30 were all changed to threonine residues to promote β-strand formation[49] (Fig. 2, shaded box). Gly24 and Ile26 were changed to valine residues to increase β-strand propensity[50] while maintaining hydrophobic character at those positions. Lastly, a leader sequence, Tyr-Gly-Gly, was placed at the helix N-terminus to aid in spectroscopic quantitation. This peptide, INPEP, demonstrated a higher helical content than the RecA N-30 peptide (see Fig. S1 in ESI†) and was assessed for its ability to inhibit RecA's ATPase activity.

The first step in both RecA-mediated SOS induction and recombinational DNA repair is the binding of RecA to ATP and ssDNA to form an active RecA–DNA filament. Active filament assembly normally results in ATP hydrolysis, which is necessary for controlling SOS induction as well as for the subsequent stages of recombinational DNA repair. ATP hydrolysis serves as a useful indicator of filament activity, and the abrogation of ATPase activity would be an important aspect of RecA inhibition. The experimental details of the *in vitro* ATPase assay have been described previously.[51,52] Briefly, the steady-state rate of ATP hydrolysis catalyzed by RecA (1 μM) in assay buffer§, at 37 °C with 3 mM ATP was compared before and after the addition of varying concentrations of peptide inhibitor. The fraction of ATPase activity remaining was plotted *versus* the inhibitor concentration to afford dose–response curves (Fig. 3).

INPEP proved to be an effective inhibitor of RecA activity with an IC_{50} of 35 μM (see Fig. 3), a ≥ 20-fold improvement over the N-30 peptide. Although the absolute IC_{50} is only modest, this inhibition is notable in the context of the monomer–monomer dissociation constant, which lies in the high nM–μM range.[38,53] Importantly, assay mixtures which contained the ATP-regeneration system and coupled NADH reporter system were not inhibited in the presence of excess INPEP, indicating the specificity of the peptide for RecA.

To improve the potency of the peptide's inhibition of RecA, a second-generation design was considered. The Cys116 residue of one RecA monomer was noted to be within disulfide bonding distance of residue 27 (Met) of the subsequent monomer (see Fig. 1). To exploit this, Met27 of INPEP was changed to a Cys residue to give INPEP-SH. We reasoned that a disulfide formed between this residue and RecA Cys116 would prevent the dissociation of INPEP-SH in the presence of competing RecA monomers, making the inhibition irreversible.

Inhibition of RecA by INPEP-SH under non-reducing conditions was both time- and dose-dependent (Fig. 4A). This result is indicative of a slow, irreversible inhibition of the ATPase activity of RecA. Because this assay was performed in the absence of reductant, disulfide bond formation was presumed to be the cause of the irreversibility. In confirmation of this hypothesis, addition of excess dithiothreitol (2 mM) to the assay mixture after complete inhibition by INPEP-SH resulted in the partial recovery of RecA ATPase activity (see Fig. S2 in ESI†).

To explore further the putative relationship between disulfide formation and inhibition by INPEP-SH, the reactivity of the cysteine thiol was modulated. First, the ATPase assay was repeated in the presence of excess DTT. Here, the assay traces did not display the curvature indicative of time-dependent irreversibility as in Fig. 4A, but rather remained linear with rates dependent on the concentration of INPEP-SH in the assay (see Fig. S2 in ESI†). This allowed for the fractional inhibition under reducing conditions to be calculated as a function of increasing concentrations of INPEP-SH (Fig. 3, ▼), which was essentially identical to the dose-response observed using INPEP (Fig. 3, ◊).

Second, to eliminate the possibility of disulfide formation between INPEP-SH and RecA, the peptidic Cys residue was

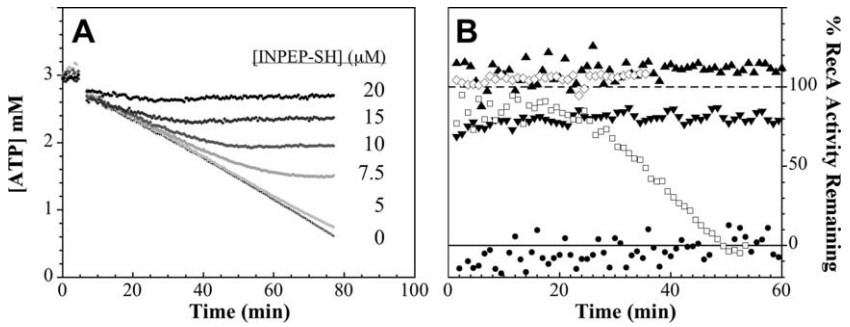

Fig. 4 **A**. *In vitro* RecA ATPase activity is irreversibly inhibited by INPEP-SH in a time and dose-dependent manner when performed without dithiothreitol reductant. The assay was initiated with addition of ssDNA and ATP after 5 min thermal equilibration (note break in data traces). Complete inhibition is seen after 15 min (20 μM), but INPEP-SH shows minimal effect relative to the uninhibited RecA control (no peptide) at 5 μM. **B**. Comparative time-dependent inhibition by 10 μM INPEP-SH without DTT (□) to same concentration of INPEP (◇), INPEP-SH + DTT (▼), INPEP-SAlk (▲), and INPEP-STP (●).

reacted with iodoacetamide to alkylate the thiol. The resultant peptide, INPEP-SAlk, inhibited RecA ATPase activity (Fig. 3, ▲) with an IC_{50} comparable to that of INPEP and INPEP-SH under reducing conditions. However, the presence of DTT in the assay buffer had no influence on the observed IC_{50} for INPEP-SAlk (data not shown).

Lastly, to facilitate disulfide formation, the electrophilicity of the cysteine residue was increased by conjugation with 2-thiopyridine to yield INPEP-STP. The 2-thiopyridone released by thiol–disulfide exchange between RecA Cys116 and INPEP-STP is resonance stabilized in neutral buffer, and thus provides the driving force for this reaction.[54] A similar derivative using 5-thio(2-nitrobenzoic acid), was used to activate a cysteine thiol in a peptide-based HIV-1 protease dimerization inhibitor.[55] The concentration-dependent RecA inactivation by INPEP-STP under non-reducing ATPase assay conditions was measured (Fig. 3, ●). By making the Cys residue on the INPEP-SH design more reactive toward free thiols, the apparent potency of the inhibitor was increased by a factor of 10.

To underscore the tunable reactivity of the peptide's thiol functional group, relative RecA activity was plotted over time for each inhibitory peptide at 10 μM (Fig. 4B). Using both INPEP (◇) and INPEP-SAlk (▲), RecA's activity was indistinguishable from uninhibited control, or 100% relative activity (dashed line), while using INPEP-SH in the presence of excess DTT (2 mM) resulted in approximately 85% relative activity (▼). Likewise, INPEP-SH without DTT present began at 85% relative RecA activity, but over the course of the assay RecA activity decreased to zero, presumably as disulfide bond formation progressed (□). Using the same concentration of INPEP-STP (●), however, the enzyme was completely inactivated at the onset of the experiment (solid line).

To assess whether the peptide was stably coupled to RecA, the assay mixture containing RecA and INPEP-SH was fractionated by SDS-PAGE and the resulting bands were analyzed by in-gel digestion MALDI-MS. Fig. 5 shows the mobility shift of the RecA band when incubated with increasing concentrations of INPEP-SH. Some higher molecular weight aggregates were also seen in the silver-stained gels, but all reverted to the original RecA band if treated with DTT prior to loading on the gel (lanes 7–10).

A similar gel containing only RecA standard and RecA reacted with INPEP-SH was submitted to UNC's Proteomics facility for in-gel digestion with chymotrypsin and subsequent MALDI-MS characterization of the fragments. Chymotrypsin was used to produce fragments where each of the three Cys residues in RecA (90, 116, or 129) were contained in individual fragments. The cysteine of RecA disulfide-bonded to the reactive Cys in INPEP-SH should then be identifiable. As anticipated, the fragment containing Cys116 was observed to form a covalent bond to INPEP-SH (see Fig. S4 in ESI†). No fragments corresponding to Cys90 or Cys129 bound to INPEP-SH were detected.

These results highlight the role of the cysteine residue in the INPEP design. Under reducing conditions, the peptide half-maximally inhibited RecA ATPase activity at 30 μM, and the iodoacetamide-inactivated and methionine peptides showed similar activities at 50 μM and 35 μM, respectively. The activated cysteine (INPEP-STP), however, showed enhanced activity (IC_{50} = 3 μM). This is likely due to an increased rate of disulfide formation, which results in virtually no time-dependence of the irreversible inhibition (Fig. 4B).

Taken together, these results demonstrate the inhibitory effect of INPEP is due to association of the peptide to the RecA protein, the effectiveness of which is enhanced by disulfide formation. This

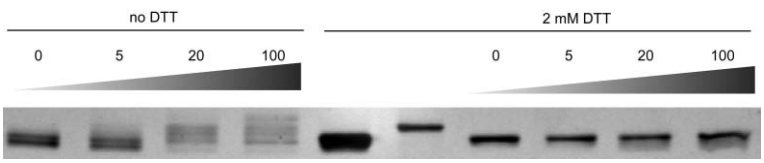

Fig. 5 SDS-PAGE of ATPase assay using INPEP-SH. Lanes 1–4 show the gel shift of the RecA band relative to reduced RecA standard (lane 5) with increasing doses of peptide inhibitor (concentrations in μM indicated above gel image) and no DTT in loading buffer. Lanes 7–10 show consolidation of bands back to reduced RecA upon addition of DTT to loading buffer. Lane 6:42.7 kDa molecular weight marker (NEB #P7702S).

peptide inhibitor, rationally designed from the RecA sequence, provides a useful lead for the development of additional peptide and small molecule inhibitors of RecA activities. Such agents ultimately have the potential to be used as stand-alone antibiotics or as adjuvants with existing antibiotics to slow the spread of antibiotic resistance.

Acknowledgements

This research was supported by the National Institutes of Health (GM58114). We also thank Dr Andrew Lee for helpful discussions and Tim Wigle and Tanarat Kietsakorn for technical assistance.

Notes and references

§ ATPase assay buffer: 25 mM Tris–HOAc, pH 7.1, 5 mM $Mg(OAc)_2$, 5% v/v glycerol with 2.3 mM PEP, 2 mM NADH, 5 U mL^{-1} LDH, 5 U mL^{-1} PK, 15 μM-nts poly(dT).

1 D. J. Diekema, B. J. BootsMiller, T. E. Vaughn, R. F. Woolson, J. W. Yankey, E. J. Ernst, S. D. Flach, M. M. Ward, C. L. Franciscus, M. A. Pfaller and B. N. Doebbeling, *Clin. Infect. Dis.*, 2004, **38**, 78–85.
2 *Microbial Threats to Health: Emergence, Detection, and Response*, ed. M. S. Smolinski, M. A. Hamburg and J. Lederberg, National Academies Press, Washington, DC, 2003.
3 G. H. Talbot, J. Bradley, J. E. Edwards, Jr., D. Gilbert, M. Scheld and J. G. Bartlett, *Clin. Infect. Dis.*, 2006, **42**, 657–668.
4 R. T. Cirz, J. K. Chin, D. R. Andes, V. de Crecy-Lagard, W. A. Craig and F. E. Romesberg, *PLoS Biol.*, 2005, **3**, e176.
5 P. L. Foster, *Mutat. Res.*, 2005, **569**, 3–11.
6 M. N. Hersh, R. G. Ponder, P. J. Hastings and S. M. Rosenberg, *Res. Microbiol.*, 2004, **155**, 352–359.
7 I. Matic, F. Taddei and M. Radman, *Res. Microbiol.*, 2004, **155**, 337–341.
8 J. W. Beaber, B. Hochhut and M. K. Waldor, *Nature*, 2004, **427**, 72–74.
9 P. J. Hastings, S. M. Rosenberg and A. Slack, *Trends Microbiol.*, 2004, **12**, 401–404.
10 C. Bisognano, W. L. Kelley, T. Estoppey, P. Francois, J. Schrenzel, D. Li, D. P. Lew, D. C. Hooper, A. L. Cheung and P. Vaudaux, *J. Biol. Chem.*, 2004, **279**, 9064–9071.
11 L. Chao, *Mutat. Res.*, 1986, **173**, 25–29.
12 R. T. Cirz, M. B. Jones, N. A. Gingles, T. D. Minogue, B. Jarrahi, S. N. Peterson and F. E. Romesberg, *J. Bacteriol.*, 2007, **189**, 531–539.
13 R. J. Fram, S. L. Mack, M. George and M. G. Marinus, *Mutat. Res.*, 1989, **218**, 125–133.
14 C. Goerke, J. Koller and C. Wolz, *Antimicrob. Agents Chemother.*, 2006, **50**, 171–177.
15 M. Herrero and F. Moreno, *J. Gen. Microbiol.*, 1986, **132**, 393–402.
16 B. M. Howard, R. J. Pinney and J. T. Smith, *J. Pharm. Pharmacol.*, 1993, **45**, 658–662.
17 P. T. Kimmitt, C. R. Harwood and M. R. Barer, *Emerging Infect. Dis.*, 2000, **6**, 458–465.
18 D. Lal, S. Som and S. Friedman, *Mutat. Res.*, 1988, **193**, 229–236.
19 C. S. Lewin and S. G. Amyes, *J. Med. Microbiol.*, 1991, **34**, 329–332.
20 S. W. Mamber, K. W. Brookshire and S. Forenza, *Antimicrob. Agents Chemother.*, 1990, **34**, 1237–1243.
21 J. L. Martinez and J. C. Perez-Diaz, *Antimicrob. Agents Chemother.*, 1986, **29**, 456–460.
22 L. S. McDaniel, L. H. Rogers and W. E. Hill, *J. Bacteriol.*, 1978, **134**, 1195–1198.
23 Y. Oda, *Mutat. Res.*, 1987, **183**, 103–108.
24 L. J. Piddock and R. N. Walters, *Antimicrob. Agents Chemother.*, 1992, **36**, 819–825.
25 T. Tamamura, M. Tsuchiya, K. Isshiki, T. Sawa, T. Takeuchi, M. Hori and N. Sakata, *J. Antibiot.*, 1988, **41**, 648–654.
26 K. Yamamoto, T. Hiramoto, H. Shinagawa and Y. Fujiwara, *Chem.-Biol. Interact.*, 1984, **48**, 145–152.
27 J. M. Gomez-Gomez, C. Manfredi, J. C. Alonso and J. Blazquez, *BMC Biol.*, 2007, **5**, 14.
28 S. Casjens, *Mol. Microbiol.*, 2003, **49**, 277–300.
29 K. A. Kline, E. V. Sechman, E. P. Skaar and H. S. Seifert, *Mol. Microbiol.*, 2003, **50**, 3–13.
30 R. T. Cirz, B. M. O'Neill, J. A. Hammond, S. R. Head and F. E. Romesberg, *J. Bacteriol.*, 2006, **188**, 7101–7110.
31 N. Haruta, X. N. Yu, S. X. Yang, E. H. Egelman and M. M. Cox, *J. Biol. Chem.*, 2003, **278**, 52710–52723.
32 A. M. Lee and S. F. Singleton, *J. Inorg. Biochem.*, 2004, **98**, 1981–1986.
33 T. J. Wigle and S. F. Singleton, *Bioorg. Med. Chem. Lett.*, 2007, DOI: 10.1016/j.bmcl.2007.04.013.
34 A. M. Lee, C. T. Ross, B. B. Zeng and S. F. Singleton, *J. Med. Chem.*, 2005, **48**, 5408–5411.
35 T. J. Wigle, A. M. Lee and S. F. Singleton, *Biochemistry*, 2006, **45**, 4502–4513.
36 R. M. Story, I. T. Weber and T. A. Steitz, *Nature*, 1992, **355**, 318–325.
37 T. Horii, N. Ozawa, T. Ogawa and H. Ogawa, *J. Mol. Biol.*, 1992, **223**, 105–114.
38 R. Masui, T. Mikawa and S. Kuramitsu, *J. Biol. Chem.*, 1997, **272**, 27707–27715.
39 T. Mikawa, R. Masui, T. Ogawa, H. Ogawa and S. Kuramitsu, *J. Mol. Biol.*, 1995, **250**, 471–483.
40 S. G. Sedgwick and G. T. Yarranton, *Mol. Gen. Genet.*, 1982, **185**, 93–98.
41 G. T. Yarranton and S. G. Sedgwick, *Mol. Gen. Genet.*, 1982, **185**, 99–104.
42 E. N. Zaitsev and S. C. Kowalczykowski, *Mol. Microbiol.*, 1998, **29**, 1317–1318.
43 M. J. de Vega, M. Martin-Martinez and R. Gonzalez-Muniz, *Curr. Top. Med. Chem.*, 2007, **7**, 33–62.
44 D. Fry and H. Sun, *Mini-Rev. Med. Chem.*, 2006, **6**, 979–987.
45 L. O. Sillerud and R. S. Larson, *Curr. Protein Pept. Sci.*, 2005, **6**, 151–169.
46 A. J. Doig, C. D. Andrew, D. A. Cochran, E. Hughes, S. Penel, J. K. Sun, B. J. Stapley, D. T. Clarke and G. R. Jones, *Biochem. Soc. Symp.*, 2001, 95–110.
47 D. A. Cochran, S. Penel and A. J. Doig, *Protein Sci.*, 2001, **10**, 463–470.
48 J. M. Scholtz, H. Qian, V. H. Robbins and R. L. Baldwin, *Biochemistry*, 1993, **32**, 9668–9676.
49 P. Y. Chou and G. D. Fasman, *Annu. Rev. Biochem.*, 1978, **47**, 251–276.
50 D. L. Minor and P.S. Kim, *Nature*, 1994, **367**, 660–663.
51 M. D. Berger, A. M. Lee, R. A. Simonette, B. E. Jackson, A. I. Roca and S. F. Singleton, *Biochem. Biophys. Res. Commun.*, 2001, **286**, 1195–1203.
52 S. W. Morrical and M. M. Cox, *Biochemistry*, 1990, **29**, 837–843.
53 S. W. Morrical and M. M. Cox, *Biochemistry*, 1985, **24**, 760–767.
54 K. Maruyama, H. Nagasawa and A. Suzuki, *Peptides*, 1999, **20**, 881–884.
55 R. Zutshi and J. Chmielewski, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 1901–1903.
56 D. J. Cline, C. Thorpe and J. P. Schneider, *Anal. Biochem.*, 2004, **335**, 168–170.

