Revisiting the S2 specificity of papain by structural analogs of Phe

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Abstract Papain characteristically has a strong preference for encoded L-aromatic amino acids (Phe > Tyr) at P2 position. We re-examined papain S2 specificity using structural analogs of Phe, in fluorogenic substrates of the series: dansyl-Xaa-Arg-Ala-Pro-Trp (Xaa = P2 residue). Kinetic analyses showed that the S2 pocket accommodates a broad spectrum of Phe derivatives. Papain is poorly stereoselective towards Dns-(D/L)-Phe-Arg-Ala-Pro-Trp and binding is not critically affected by replacement of the benzyl ring by the non-aromatic lateral chain of cyclohexylalanine. The K_m was significantly improved by mono- and di-chlorination of Phe, or by its substitution by an electronegative group-like NO_2, but the specificity constant was unchanged. Shortening or lengthening the side chain by adding or removing a methylene group impairs the P2/S2 interactions significantly, as do constrained structural analogs of Phe. Incorporation of benzyl-substituted phenylalanyl amino acid could help to design peptide-derived inhibitors with greater affinity and bioavailability.

Key words: Papain; Cysteine proteinase; Substrate specificity; Phenylalanine analog

1. Introduction

Papain, obtained from the latex of *Carica papaya* (EC 3.4.22.2), is the archetype of the C1 family (clan CA), the best known family of cysteine proteinases [1]. The enzyme is a monomeric endopeptidase whose active site lies at the interface between the R- and L-domains [2]. Two conserved catalytic residues, the highly nucleophilic Cys-25 and His-159, are mainly involved in its activity, which is optimal at slightly acidic pH [3]. Other residues, such as Asn-175 which is hydrogen-bonded to the protonated side chain of His-159, keeping the imidazolium ring in a favorable orientation, and Gln-19 which takes part in the formation of the oxianion hole, play critical roles in catalysis [4]. The substrate forms hydrogen bonds with residues of the active site cleft and binds in an extended conformation [5]. The crystal structure of papain in complex with a chloromethyl ketone substrate analog reveals that the P1 side chain faces the solvent, whereas the P2 side chain contacts the papain surface inside an enclosed cavity [6]. Several approaches have been used to study papain specificity since the original work by Schechter and Berger [7], including kinetic and structural analyses of the hydrolysis of synthetic ester or amide substrates and protein substrates [8–12], as well as site-directed mutagenesis of residues of the catalytic site [13–15]. It is now clearly established that the specificity of papain is predominantly determined by P2/S2 interactions. Unlike lysosomal cathepsin B, papain has a pronounced preference for bulky hydrophobic or aromatic residues such as Phe in P2 position, which is sandwiched between the side chains of Val-133 and Val-157 at subsite S2. This preference is thought to be due to the presence of a seryl residue at position 205 in the bottom of the S2 binding pocket, instead of a glutamic acid in cathepsin B [16].

However, most of these kinetics studies have been performed using encoded amino acids. We have now introduced structural analogs of Phe into position P2 of fluorogenic substrates with resonance energy transfer properties and used these substrates to further investigate the S2 subsite specificity of papain. Determination of the ability of papain-like cysteine proteinases to accommodate unencoded amino acids in their S2 binding pocket may provide valuable information for the improving specificity and/or bioavailability of inhibitors of papain-like enzymes.

2. Material and methods

Unless otherwise stated, all Fmoc-protected amino acids were of the L-configuration and were purchased from Neosystem (Strasbourg, France) or Advanced ChemTech Europe (Brussells, Belgium). Dicyclohexylcarbodiimide was obtained from Sigma-Aldrich (St Quentin le Fallavier, France) and hydroxybenzotriazole (HOBt) from Novabiochem (FranceBiochem, Meudon, France). Papain was purchased from Boehringer-Mannheim (Germany). Z-Phe-Arg-pNA was from Novabiochem. L-3-carboxy-trans-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane (E-64) and DL-dithiothreitol (DTT) were from Sigma-Aldrich. All other reagents were of analytical grade.

2.1. Peptide synthesis

Substrates were synthesized as peptidyl amides by Fmoc solid-phase chemistry [17], using a Rink Amide MBHA resin (0.1 mmol equivalent) (Novabiochem). The first four residues RAPW (10 eq.) were coupled on an automated solid phase peptide synthesizer (ABI model 431A, Perkin Elmer, Roissy, France). The resin was then divided into portions and various structural analogs of Phe (see Fig. 1) were added to this 4mer sequence. Coupling was done manually for each amino acid (4 eq.), in the presence of DCCI (4 eq.) and HOBt (2 eq.) in dichloromethane. The N-terminus was deprotected and peptides were dansylated by incubating them with dansyl chloride (3 eq.) in 0.2 M carbonate-bicarbonate buffer, pH 9.5 for 24 h [18]. Each step was checked by colorimetric assays with ninhydrin (Kaiser test). Peptidyl amides were deprotected, cleaved from the resin and purified by semi-preparative reverse phase chromatography (Aquapore octyl 20 μm column, Brownlee-Perkin-Elmer) using a 30 min linear (0–90%) acetonitrile gradient in 0.1% trifluoroacetic acid. Peptides were checked for homogeneity by analytical RP-HPLC (Brownlee C18 OD 300 column) using the elution conditions indicated above and then ana-

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Abbreviations: Cha, cyclohexyl-alanine; Phg, phenylglycine; Hof, homophenylalanine; 4-Cl-Phe, 4-chloro-phenylalanine; 3,4-Cl_2-Phe, 3,4-dichloro-phenylalanine; 4-NO_2-Phe, 4-nitro-phenylalanine; Aic, 2-amino indane carboxylic acid; Tic, (3S)1,2,3,4-tetrahydro isoquinoline 3-carboxylic acid; AMC, 7-amino-4-methyl-coumarin; DCCI, dicyclohexylcarbodiimide; Dns, dansyl; HOBt, hydroxybenzotriazole; MALDI-TOF, matrix assisted laser desorption/ionization-time-of-flight; pNA, para-nitroanilide; Rink amide MBHA resin, (4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl-phenoxyacetamido-norleucyl)-4-methylbenzhydrylamine) resin

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lyzed by MALDI-TOF mass spectroscopy (Brüker). The experimental data were compared to theoretical molecular weights (MacPromass software, Beckman Research Institute). Results are: (I) calculated molecular weight: 908.1, found molecular weight: 908.5; (II) 908.1, 908.9: (III) 914.1, 915.0: (IV) 894.1, 895.4: (V) 922.1, 922.6: (VI) 925.1, 925.1: (VII) 943.1, 943.6: (VIII) 978.1, 978.6: (IX) 953.1, 953.3: (X) 920.1, 921.9: (XI) 920.1, 921.5.

2.2. Kinetic measurement

Papain, in assay buffer (0.1 M phosphate buffer, pH 6.0, containing 2 mM DTT and 1 mM EDTA), was activated for 5 minutes at 37°C prior to kinetic measurements. The enzyme active site was titrated by E64, using Z-Phe-Arg-AMC as substrate [19].

2.2.1. Determination of k_{cat}/K_m. The hydrolysis of the intramolecularly quenched fluorogenic substrates was monitored with the excitation monochromator set at the wavelength for tryptophan absorption (290 nm) and the emission monochromator set at the wavelength for dansyl emission (550 nm) (Kontron SFM 25 spectrofluorimeter). Second order rate constants (k_{cat}/K_m) were measured by carrying out the experiments under pseudo first order conditions (substrate concentrations: 5–10 μM) and running Enzfitter software (Biosoft, Cambridge, UK) as described elsewhere [20]. Experiments were performed in triplicate and the data are reported as means ± S.D.

2.2.2. Determination of the Michaelis constant (K_m). Enzyme assays were carried out with two competing substrates, whose hydrolysis products could be measured independently. Under these mixed alternative substrates conditions, each substrate acted as a competitive inhibitor of the other (see Eq. 1) [21], and K_m values of intramolecularly quenched fluorogenic substrates were obtained by measuring the dissociation constant (K_i) towards a chromogenic substrate of papain. Assays were carried out by adding papain (13 nM) to a mixture of Z-Phe-Arg-pNA (200 μM) ($K_m = 140$ μM) [9] and a fluorogenic Phe analog containing peptide (0–200 μM). The hydrolysis of Z-Phe-Arg-pNA was monitored at 410 nm (spectrophotometer Hitachi U-2001) with less than 5% of Z-Phe-Arg-pNA hydrolyzed. The relative velocity of the enzymatic reaction is described by the following equation [21]:

$$v_i/v_o = (K_m + S)/(K_m(1 + I/K_i) + S). \quad (1)$$

with: v_i: initial velocity at a given substrate concentration with dansylated peptide, v_o: initial velocity at the same substrate concentration without dansylated peptide, K_m: Michaelis constant of the substrate, S: chromogenic substrate concentration, I: dansylated peptide concentration.

The K_i value corresponds to the Michaelis constant of the dansylated Phe analog containing substrate used as a competitor.

2.3. RP-HPLC analysis

Papain (0.275 μM) was incubated with peptides (100 μM) in activation buffer at 37°C for 1 h (final volume: 30 μl). The reaction was stopped by adding 100 μl ethanol to precipitate the papain. The supernatants containing the native peptide and/or its proteolytic fragments were evaporated and redissolved in 0.1% TFA. An aliquot of each sample was fractionated by reverse phase chromatography on a C18 OD 300 column (Brownlee), using a 20 min linear (0–90%) gradient of acetonitrile in 0.1% TFA at a flow rate of 0.2 ml/min. Proteolysis products were identified by comparison with native peptidyl amides and the elution profiles were analyzed by Spectacle software (ThermoQuest, les Ulis). Cleavage sites were located by N-terminal sequencing (ABI 477A sequencer, Perkin-Elmer).

3. Results and discussion

We have carried out a systematic kinetic characterization of the S2 binding site specificity of papain using a series of substrates with resonance energy transfer properties and containing structural analogs of phenylalanine in position P2. These substrates were N-terminally dansylated (Dns) and had a common framework (Dns-Xaa-Arg-Ala-Pro-Trp), where Xaa = Phe analog. Residues at P1, P1′ and P2′ were chosen, based on previous reports [9,20]. Determination of k_{cat}/K_m values were based on the transfer of excitation energy occurring between the Trp indole ring (emission wavelength = 334 nm) and the dansyl group (absorption band = 335 nm), due to their spatial proximity in the intact peptide [22]. The papain-catalyzed cleavage of fluorescent substrate can be therefore monitored by the increase in fluorescence at 340 nm or by the decrease in fluorescence at 550 nm (Dns emission). The enzymatic reactions were monitored by recording the decrease in fluorescence at 550 nm to avoid background fluorescence from the Trp residues in papain (see Section 2). The enzyme concentration has no significant effect on the dansyl fluorescence under these conditions [11]. Low substrate concentrations (micromolar range) were used ([S] ≪ K_m) to obtain first order conditions [11,23].

Two hydrolysis products were generated from each peptide substrate upon incubation with papain (Fig. 2). The one which absorbed at 320–330 nm was the N-terminal dansylated fragment (Dns-XaaR). Its retention time depended on the nature of the amino acid Xaa. The second peak had the same retention time (11.54 min) for compounds I–XI and was the C-terminal APW (Fig. 2). Cleavage occurred at the Arg-Ala bond, demonstrating that introduction of Phe structural analogs in P2 does not affect the substrate positioning in

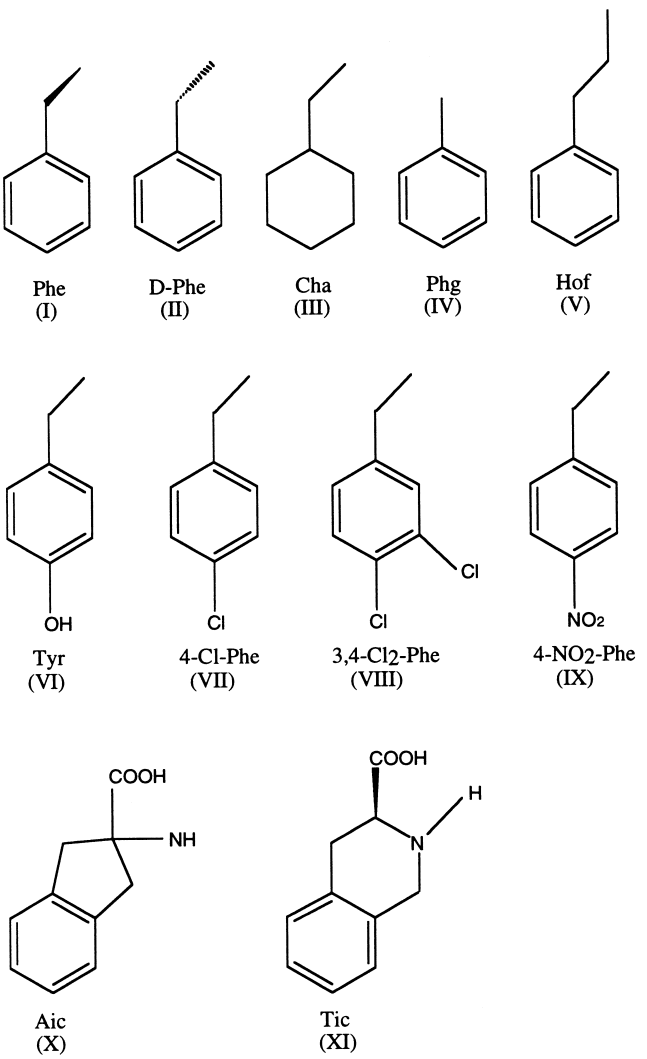


Fig. 1. Molecular structure of the Xaa side chain of substrates I–XI of the series: dansyl-Xaa-Arg-Ala-Pro-Trp. (For the listed code corresponding to Phe analogs: see abbreviations).

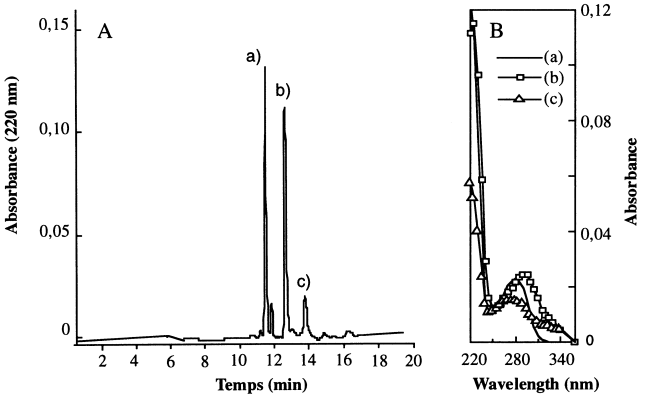


Fig. 2. Reverse phase HPLC and absorption spectra of the products of papain-catalyzed hydrolysis of Dns-Aic-Arg-Ala-Pro-Trp. (A) Peaks a and b were proteolysis products, while peak c was identified as the native peptide (compound X). (B) Peak a corresponds to the C-terminal peptidyl-amide Ala-Pro-Trp, while peak b is the N-terminal fragment Dns-Aic-Arg.

the active site. No further cleavage was observed even after completion of the hydrolysis.

The second order rate constants (k_{cat}/K_m) and Michaelis constants (K_m) show that the papain S2 pocket can accommodate most of Phe derivatives in position P2 (Table 1). Dns-(L)-Phe-Arg-Ala-Pro-Trp and Dns-(D)-Phe-Arg-Ala-Pro-Trp bound to papain with the same K_m. However, the index of stereochemical selectivity I_{ss} ($I_{ss} = (k_{cat}/K_m)_L/(k_{cat}/K_m)_D$) for the two enantiomeric forms was 6.4, indicating that only the efficiency of the chemical reaction is affected by the inversion of chirality. Although the S2 subsite of papain has long been claimed to be highly stereoselective [7], some studies have shown that the P2/S2 stereoselectivity may depend greatly on the nature and chemical reactivity of the substrate. Hanzlik et al. [24] reported an I_{ss} of eight for the papain-catalyzed hydrolysis of N-acetyl-Phe-Gly-ONp, whereas the I_{ss} for corresponding *p*-nitroanilide derivatives (*N*-acetyl-Phe-Gly-pNA) was 330 [25]. A small, 5-fold, difference in K_i values was also reported for enantiomeric forms of peptidyl aldehydes [26]. According to Brocklehurst and co-workers, the α-benzyl side chain of L-Phe fits very close to the side chains of Val-133 and Val-157 in the S2 subsite of papain, while that of the D-form is closer to the protein-solvent interface [27].

An aromatic ring on the Xaa side chain at position P2 is not essential for interaction, since the non-polar bulky group of Cha still binds to papain without any critical change in the specificity constant (Table 2). This Cha containing substrate has an even better K_m than compound VI, which has a Tyr in P2 (Table 1). Aromatic rings, such as the benzyl group of Phe or the phenolic group of Tyr which fit tightly into the S2 subsite, may therefore be replaced by a saturated 6-carbon ring, which is probably in a chair-like conformation and fits into the S2 pocket as does a plane-conjugated ring.

But the number of methylene groups of the side chain of Phe is more important for the P2/S2 interaction. Deletion or addition of a $-CH_2$ between the α-carbon and the benzyl group (compounds IV and V) dramatically decreases k_{cat}/K_m (Phe ≫ Hof > Phg) (Table 1,Table 2), due to an increase in K_m and a decrease in k_{cat}. This means that the correct insertion of the aromatic ring between Val-133 and Val-157 in the S2 binding pocket of papain depends greatly on the length of the carbon backbone of the side chain (i.e. the distance between the asymmetric carbon and the aromatic ring).

The mono- or di-substitutions by a chloride atom (compounds VII and VIII) are at variance with these results, with significantly decreased K_m values, though the k_{cat}/K_m were not affected (Table 1,Table 2). Para-substitution of the Phe side chain by NO_2 results in a similar decrease in the Michaelis constant (Table 1). But we could not measure the specificity constant k_{cat}/K_m for this compound (compound IX) because the nitro group interfered with the energy transfer between Dns and Trp. Halogenation or adding a strong electronegative NO_2 group to the α-benzyl ring of the side chain therefore favors P2/S2 interactions.

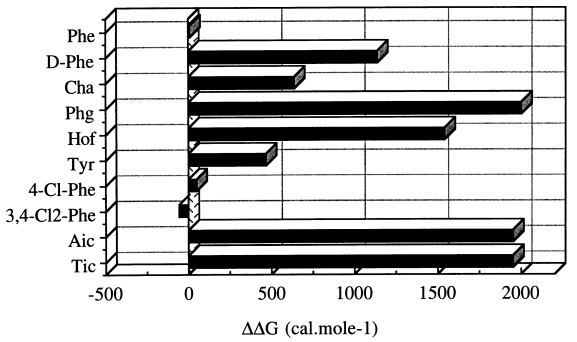
Constrained forms of Phe (Aic, Tic) are poorly accepted within the S2 subsite of papain. This results in a decrease in k_{cat}/K_m and an increase in Michaelis constants, compared to the Phe containing substrate (Tables 1 and 2). The crystal structure of peptidylchloromethylketone-complexed papain [2] shows that the lack of rotational freedom produced by an additional covalent bond linking the side chain to the backbone, prevents the aromatic ring fitting correctly in the S2 binding pocket.

Table 1
Kinetic parameters (k_{cat}/K_m, K_m) for the hydrolysis of dansylated fluorogenic substrates by papain

Dns-Xaa-RAPW		Papain		
N°	Xaa	k_{cat}/K_m [a] (mM^{-1}/s)	K_m (μM)	$K_m/(K_m)_{ref}$ [b]
I	Phe	1228 ± 195	44 ± 13	1.0
II	D-Phe	192 ± 36	45 ± 11	1.0
III	Cha	432 ± 159	95 ± 21	2.2
IV	Phg	47 ± 3	607 ± 98	13.8
V	Hof	100 ± 6	525 ± 39	11.9
VI	Tyr	569 ± 130	196 ± 25	4.5
VII	4-Cl-Phe	1140 ± 176	14 ± 2	0.3
VIII	3,4-Cl_2-Phe	1354 ± 178	20 ± 4	0.5
IX	4-NO_2-Phe	n.d.	23 ± 4	0.5
X	Aic	51 ± 10	212 ± 74	4.8
XI	Tic	51 ± 5	318 ± 18	7.2

Experiments were carried out in 0.1 M phosphate buffer, pH 6.0, containing 2 mM DTT and 1 mM EDTA, as described in Section 2. The data corresponded to triplicate experiments and were averaged ± S.D.
[a]Measurements of second order rate constants k_{cat}/K_m have been performed under pseudo first order conditions, according to Ménard et al. [11], running the Enzfitter software (Biosoft, Cambridge, UK).
[b]$(K_m)/(K_m)_{ref}$: $(K_m)_{Dns-Xaa-RAPW}/(K_m)_{Dns-FRAPW}$ ratio.
n.d., non determined.

Table 2
Energetic contribution of Phe analogs residues for binding to papain



The strenght of individual contribution (ΔΔG) of Phe structural analogs in the P2 position of the dansylated substrates of the series Dns-Xaa-RAPW for binding to the S2 subsite of papain was determined, according to the equation $\Delta\Delta G = -RT\ ((\ln (k_{cat}/K_m)_{Dns-Xaa-RAPW}/\ln (k_{cat}/K_m)_{Dns-FRAPW})$, by using L-(Phe) as reference residue.

Our data thus show that papain accommodates a broad spectrum of Phe derivatives in its S2 pocket. However mono- and di-halogenations of the benzyl group, or para-substitution by NO_2, give the best interacting compounds. These findings can now be used to design pseudo peptidyl inhibitors, with a benzyl-substituted phenylalanyl amino acid at P2, that are more stable in vivo, while retaining an improved affinity [28].

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References

[1] Rawlings, N.D. and Barrett, A.J. (1993) Biochem. J. 290, 205–218.
[2] Drenth, J., Jansonius, J.N., Koekoek, R. and Wolthers, B.G. (1971) Adv. Protein Chem. 25, 79–115.
[3] Storer, A.C. and Ménard, R. (1994) Methods Enzymol. 244, 486–500.
[4] Ménard, R., Carrière, J., Laflamme, P., Plouffe, C., Khouri, H.E., Vernet, T., Tessier, D.C., Thomas, D.Y. and Storer, A.C. (1991) Biochemistry 30, 8924–8928.
[5] Turk, B., Turk, V. and Turk, D. (1997) Biol. Chem. Hoppe Seyler 378, 141–150.
[6] Drenth, J., Kalk, K.H. and Swen, H.M. (1976) Biochemistry 15, 3731–3738.
[7] Schechter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162.
[8] Koga, H., Yamada, H., Nishimura, Y., Kato, K. and Imoto, T. (1990) J. Biochem. 168, 976–982.
[9] Tchoupé, J.R., Moreau, T., Gauthier, F. and Bieth, J.G. (1991) Biochim. Biophys. Acta 1076, 149–151.
[10] Garcia-Echeverria, C. and Rich, D.H. (1992) FEBS Lett. 297, 100–102.
[11] Ménard, R., Carmona, E., Plouffe, C., Brömme, D., Konishi, Y., Lefèvre, J. and Storer, A.C. (1993) FEBS Lett. 328, 107–110.
[12] Serveau, C., Juliano, L., Bernard, P., Moreau, T., Mayer, R. and Gauthier, F. (1994) Biochimie 76, 153–158.
[13] Khouri, H.E., Vernet, T., Ménard, R., Parlati, F., Laflamme, P., Tessier, D.C., Gour-Salin, B., Thomas, D.Y. and Storer, A.C. (1991) Biochemistry 30, 8929–8936.
[14] Altschuh, A., Tessier, D.C. and Vernet, T. (1994) Protein Eng. 7, 769–775.
[15] Ménard, R., Khouri, H.E., plouffe, C., Dupras, R., Ripoll, D., Vernet, T., Tessier, D.C., Laliberté, F., Thomas, D.Y. and Storer, A.C. (1990) Biochemistry 29, 6706–6713.
[16] Musil, D., Zucic, D., Turk, D., Engh, R.A., Mayr, I., Huber, R., Popovic, T., Turk, V., Towatari, T., Katunuma, N. and Bode, W. (1991) EMBO J. 10, 2321–2330.
[17] Atherton, E. and Sheppard, R.C. (1989) Solid Phase Peptide Synthesis. A practical approach, IRL press, Oxford.
[18] Gray, W.R. (1967) Methods Enzymol. 11, 139–151.
[19] Barrett, A.J., Kembhavi, A.A., Brown, M.A., Kirschke, H., Knight, C.G., Tamai, M. and Hanada, K. (1982) Biochem. J. 201, 189–198.
[20] Serveau, C., Lalmanach, G., Juliano, M.A., Scharfstein, J., Juliano, L. and Gauthier, F. (1996) Biochem. J. 313, 951–956.
[21] Segel, I.H. (1975) Enzyme Kinetics, John Wiley and Sons, New York.
[22] Fairclough, R.H. and Cantor, C.R. (1978) Methods Enzymol. 48, 347–379.
[23] El Moujahed, A., Gutman, N., Brillard, M. and Gauthier, F. (1990) FEBS Lett. 265, 137–140.
[24] Hanzlik, R.P., Jacober, S.P. and Zygmunt, J. (1991) Biochim. Biophys. Acta 1073, 33–42.
[25] Kowlessur, D., Thomas, E.W., Topham, C.M., Templeton, W. and Brocklehurst, K. (1990) Biochem. J. 266, 653–660.
[26] MacKenzie, N.E., Grant, S.K., Scott, A.I. and Malthouse, J.P.G. (1986) Biochemistry 25, 2293–2298.
[27] Patel, M., Kayani, I.S., Mellor, G.W., Sreedharan, S., Templeton, W., Thomas, E.W., Thomas, M. and Brocklehurst, K. (1992) Biochem. J. 281, 553–559.
[28] Dragovich, P.S., Webber, S.E., Babine, R.E., Fuhrman, S.A., Patick, A.K., Matthews, D., Reich, S.H., Marakovits, J.T., Prins, T.J., Zhou, R., Tikhe, J., Littlefield, E.S., Bleckman, T.M., Wallace, M.B., Little, T.L., Ford, C.E., Meador, J.W., Ferre, R.A., Brown, E.L., Binford, S.L., DeLisle, D.M. and Worland, S.T. (1998) J. Med. Chem. 41, 2819–2834.