Volume 259, number 1, 75–78 FEB 07926 December 1989

A synthetic peptide substrate specific for casein kinase-1

Patrizia Agostinis, Lorenzo A. Pinna*, Flavio Meggio*, Oriano Marin*, Jozef Goris, Jackie R. Vandenheede and Wilfried Merlevede

Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit te Leuven, Belgium and **Dipartimento di Chimica Biologica e Centro Studi Fisiologia Mitocondriale del Centro Nazionale delle Ricerche, Università di Padova, Italy*

Received 18 October 1989

The synthetic peptide, Asp-Asp-Asp-Glu-Glu-Ser-Ile-Thr-Arg-Arg, derived from the phosphorylation site of casein kinase-1 (CK-1) in β-casein A^2, is readily phosphorylated by CK-1, but not by casein kinase-2 (CK-2), cyclic AMP-dependent protein kinase, protein kinsae C, phosphorylase kinase and protein kinase F_A. Phosphorylation by CK-1 occurs only at Ser-6, Thr-8 being unaffected. The K_m for the peptide is higher (1 mM) than for β-casein A^2 (40 μM), while the V_{max} is quite comparable. This is the first synthetic peptide substrate for CK-1 described so far, and can be used for the rapid and specific estimation of CK-1 activity in crude extracts.

Casein kinase-1; Peptide phosphorylation; Protein kinase specificity

1. INTRODUCTION

Casein kinases are ubiquitous cyclic nucleotide-independent and Ca^{2+}/calmodulin-insensitive protein kinases with an in vitro preference for acidic proteins such as casein or phosvitin over histones [1]. They are distinct from the mammary gland enzymes which phosphorylate casein in vivo [2]. The casein kinases catalyze the phosphorylation of a variety of proteins involved in the regulation of cellular functions, including glycogen and lipid metabolism, gene expression and protein synthesis (reviewed in [1,3]). There are two classes of casein kinases, type-1 (CK-1) and type-2 (CK-2), with a different structure, nucleotide and peptide substrate specificity and responsiveness to various effectors such as heparin and polycations [1,3]. The local specificity determinants of CK-2 have been elucidated with the aid of synthetic peptides constructed according to the substrate phosphorylation sites [4–9]. The crucial determinant for efficient catalysis by CK-2 consists of Ser- or Thr-residues followed by a number of negatively charged amino acids, among which the location at position +3 appears to play a crucial role [4]. The amino acid sequence of the phosphorylation sites in the protein substrates supports the idea that acidic residues preceding rather than following the target amino acid are important determinants for phosphorylation by CK-1 [3]. However, no systematic studies on the site-specificity of CK-1 have been undertaken yet, and no synthetic peptide susceptible to phosphorylation by this enzyme was known so far.

Correspondence address: P. Agostinis, Afdeling Biochemie, Faculteit Geneeskunde, Campus Gasthuisberg, Herestraat, B-3000 Leuven, Belgium

Recent studies on the possible hormonal regulation of CK-2 [10,11] have exploited the use of a specific synthetic peptide substrate of CK-2 for monitoring its activity in crude cellular extracts. Specific substrates for casein kinases would be especially useful in the study of their regulation in the presence of other casein-phosphorylating activities. Therefore we searched for a substrate phosphorylated specifically by CK-1.

In the present paper we discuss a new synthetic peptide substrate for CK-1 which is not phosphorylated by CK-2, kinase F_A, cyclic AMP-dependent protein kinase, phosphorylase kinase and protein kinase C. This peptide, which is reminiscent of the CK-1 phosphorylation site in β-casein A^2, can also be used for an easy and rapid monitoring of CK-1 activity during the early steps of purification from rat liver.

2. MATERIALS AND METHODS

2.1. *Materials*

The synthetic peptide, Asp-Asp-Asp-Glu-Glu-Ser-Ile-Thr-Arg-Arg, referred to as 'βC-peptide' in this paper, was obtained from Multiple Peptide System (San Diego, USA) and from Cambridge Research Biochemicals (Cambridge, England). The purity of the synthetic peptides was more than 95%, guaranteed for the latter product by amino acid analysis and fast atomic bombardment mass spectrometry. The results obtained with both synthetic peptide preparations were identical. The peptides Ser-Glu-Glu-Glu-Glu-Glu, Arg-Arg-Ala-Ser-Val-Ala and Gly-Ser-Orn-Orn-Orn-Orn-Orn-Orn-Tyr were kindly provided by Dr F. Marchiori (Padova, Italy). Whole casein and β-casein A^2 [12], rabbit skeletal muscle phosphorylase *b* [13], ATP, Mg-dependent (AMD) phosphatase [14], phosphorylase kinase [15] and protein kinases F_A from rabbit skeletal muscle [16] were prepared according to the published methods. The catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle [17] was kindly provided by Dr B.A. Hemmings (Basel, Switzerland) and bovine brain protein kinase C [18] by Dr P.J. Parker (London). CK-1 and CK-2 were purified from rat liver cytosol essentially as in [19] ex-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

cept for CK-2, the enzyme purity of which was improved by an additional Mono-Q FPLC step. Dowex 1-X8 ion exchange resin was obtained from Bio-Rad; radiolabeled ATP from the Radiochemical Centre, Amersham.

2.2. *Assays*

Different procedures were adopted for assaying the protein kinases. The phosphorylation reactions were stopped by spotting an aliquot of the incubation mixture onto square papers of either Whatman P81 in the case of peptides containing at least two basic amino acids, or Whatman ET31 in the case of phosphorylase *b* and casein and subsequent immersion in acetic acid [20] or trichloroacetic acid [21], respectively. Controls with either substrates or protein kinases alone were performed at the same time, and subtracted from the substrate phosphorylation data. When the acidic peptide, Ser-Glu-Glu-Glu-Glu-Glu, was used, the reaction was stopped with 30% acetic acid and the labeled substrate separated from [^{32}P]ATP through anion exchange (1 ml Dowex 1-X8 column) as described in [22]. Unless indicated otherwise, the reaction time was 15 min at 30°C and the final volume 25 μl. The catalytic subunit of the cyclic AMP-dependent protein kinase was assayed in the presence of 70 mM Mes (pH 6.5), 200 μM [γ-^{32}P]ATP (4000 cpm/pmol), 15 mM $MgCl_2$ and 200 μM Arg-Arg-Ala-Ser-Val-Ala as control substrate. The protein kinase C reaction mixture contained 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 200 μM [γ-^{32}P]ATP (4000 cpm/pmol), 0.5 mM Ca^{2+}, 50 μg/ml phosphatidylserine, 5 μg/ml diolein and 0.8 mM of the synthetic peptide Gly-Ser-Orn-Orn-Orn-Orn-Orn-Orn-Tyr as reference substrate. Phosphorylase kinase reactions were carried out in the presence of 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl^{2+}$, 200 μM [γ-^{32}P]ATP (4000 cpm/pmol), 10^{-5} M cyclic AMP and 5 mg/ml phosphorylase *b*. CK-1 and CK-2 were assayed at 37°C in a mixture containing 60 μM [γ-^{32}P]ATP (300–1000 cpm/pmol), 50 mM Tris-HCl (pH 7.4), 12 mM $MgCl^{2+}$, 100 mM NaCl using 2.5 mg whole casein per ml as reference substrate. The kinase F_A was tested for its ability to activate the inactive AMD phosphatase as a control substrate [23]; 1 unit of kinase F_A activity is that amount of enzyme which produces 1 unit of phosphatase activity from the inactive AMD phosphatase. βC-peptide kinase activity was measured with the different purified kinases under the same conditions with 2.9 mM βC-peptide as the substrate with the exception of kinase F_A whose activity toward the βC-peptide was measured in a medium containing 50 mM Tris-HCl (pH 7.4), 12 mM $MgCl_2$, 150 μM [γ-^{32}P]ATP (500 cpm/pmol). The radiolabeled phosphoamino acids phosphorylated by CK-1 in the βC-peptide were isolated and identified by a 4 h hydrolysis in 6 N HCl at 110°C followed by high-voltage electrophoresis at pH 1.9 and quantified as in [24]. Kinetic constants were calculated from double reciprocal plots constructed from initial rate measurements fitted to the Michaelis-Menten equation.

2.3. *Phosphocellulose chromatography of casein kinase-1 and -2*

The livers (±30 g from 3 rats) were homogenized in 3 vols of buffer A containing 40 mM glycylglycine, 3 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM benzamidine, 0.3 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM β-mercaptoethanol, 0.25 M sucrose and 5% glycerol at pH 7.4. The high speed supernatant (35 min at 50 000 rpm) was brought to 0.25 M NaCl and absorbed batch-wise to 30 ml of phosphocellulose equilibrated in buffer B containing 0.05 M Tris-HCl, 0.25 M NaCl, 0.5 mM PMSF, 0.5 mM benzamidine, 0.1 mM tosyl-lysylchloromethane (TPCK), 0.01% Brij at pH 7.4, and washed with 300 ml of buffer B followed by 300 ml of buffer B containing 0.35 M NaCl. The slurry was poured into a column, washed with 50 ml of 0.35 M NaCl in buffer B and eluted with 350 ml of a linear 0.35–1 M NaCl salt gradient in the same buffer (see section 3). Neither the activity nor the chromatographic profile of the casein kinases were affected by pre-treatment of the animal with glucagon. The casein kinase activity of the fractions (25 μl) was assayed by incubation with 2.5 mg whole casein per ml for 15 min at 37°C in 0.1 ml of a medium containing: 50 mM Tris-HCl at pH 7.4, 12 mM $MgCl^{2+}$ and 12 μM [γ-^{32} P]ATP (380 cpm/pmol). The reaction was stopped by adding 10% trichloroacetic acid as described in [19]. Phosphorylation of the βC-peptide (2.9 mM) and of the peptide, Ser-Glu-Glu-Glu-Glu-Glu (1.2 mM), was assayed by incubating 10 μl aliquots of the column fractions at 37°C for 20 min in a final vol. of 25 μl containing 50 mM Tris-HCl at pH 7.4, 12 mM $MgCl^{2+}$ and 60 μM [γ-^{32} P]ATP (700 cpm/pmol).

3. RESULTS AND DISCUSSION

Although the structural determinants of CK-1 are still nor clear, it is supposed that acidic residues upstream from the phosphorylatable amino acid are required for the target recognition by this enzyme. Such an assumption is based mainly on the structure of the sites which are most readily phosphorylated by CK-1 in casein fractions and consists of seryl residues located on the C-terminal side of acidic clusters containing both phosphoserines and glutamic acid residues [25,26]. Table 1 shows the sites phosphorylated by CK-1 in different casein variants. The relevant role of both residues is further substantiated by the finding that previous dephosphorylation of the 5–21 tryptic fragment of α_{S2}-casein containing the phospho-acceptor site, Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser_{13}-Ile-Ile, impairs the phosphorylation of Ser_{13} by CK-1 [26]. In analogy it has been shown recently that phosphorylation of glycogen synthase by cyclic AMP-dependent protein kinase is a prerequisite for subsequent phosphorylation by CK-1 [27]. It is conceivable, however, that carboxyl groups can substitute for the phosphorylated amino acid residues as specificity determinants for CK-1, since phosphoamino acids are absent in some targets of CK-1 [28,29]. However, since the peptide, Glu-Glu-Glu-Glu-Glu-Ser, is not affected at all by CK-1 (unpublished results), we have assumed that additional residue(s) located on the C-terminal side of the serine could also be required besides the N-terminal acidic ones in order to construct a suitable peptide substrate for CK-1.

Based on the above assumptions the peptide, Asp-Asp-Asp-Glu-Glu-Ser-Ile-Thr-Arg-Arg (βC-peptide), has been synthesized which is closely reminiscent of the 17–26 segment of β-casein A^2 (see table 1) surrounding Ser-22, i.e. one of the most efficient phospho-acceptor sites for CK-1 [25]. To facilitate the peptide synthesis by traditional methods the 3 endogenously phosphorylated

Table 1

Phosphorylation sites of CK-1 in casein variants

Casein variants	Sites phosphorylated
α_{S2}-casein	-His-Val-Ser(p)-Ser(p)-Ser(p)-Glu-Glu-**Ser**-Ile-Ile-Ser(p)-
α_{S2}-casein	-Leu-Ser(p)-Thr-Ser(p)-Glu-Glu-Asn-**Ser**-Lys-Lys-Thr-
β-casein A^2	-Ser(p)-Ser(p)-Ser(p)-Glu-Glu-**Ser**-Ile-Thr-Arg-Ile-

S(p) denotes endogenously phosphorylated serine residues. Those affected by CK-1 are indicated in bold type. Carboxyl residues and endogenously phosphorylated residues are underlined. Amino acid sequences as in [3,26]

Table 2

Activity of some protein kinases toward the synthetic peptide Asp-Asp-Asp-Glu-Glu-Ser-Ile-Thr-Arg-Arg (βC-peptide)

Protein kinase	Control substrate	^{32}P incorporated (pmol)	
		Control	βC-peptide
Casein kinase-1	whole casein	277	168
Casein kinase-2	whole casein	595	<1
Protein kinase C	Gly-Ser-(Orn)$_6$-Tyr	412	<1
Cyclic AMP-dependent protein kinase	(Arg)$_2$-Val-Ser-Val-Ala	245	<1
Phosphorylase kinase	phosphorylase *b*	179	<1
Protein kinase F_A	AMD phosphatase	2000 (U*)	<1

* 1 unit of kinase F_A produces 1 unit of active AMD phosphatase in a 10 min preincubation at 30°C [23]

N-terminal serines were replaced by aspartic acid, which closely resembles phosphoserine, while a second arginine was added at the C-terminal end instead of isoleucine in order to facilitate the binding of the peptide to the phosphocellulose paper employed for the assay of kinase activity [20]. The βC-peptide could be phosphorylated by CK-1, the phosphorylation reaction proceeding linearly for at least 15 min, and the kinase reaction could be completely accounted for by the modification of the serine residue which is also affected in intact β-casein A^2 within the similar peptide sequence, based on the amino acid analysis of the acidic hydrolysate of the ^{32}P-product of the reaction (not shown). The K_m value of the peptide is 1 mM, i.e. 25-fold higher than that of β-casein A^2 (40 μM), but still quite comparable with the K_m values reported for peptide substrates of CK-2 [4,6]. However, the maximum phosphorylation velocity of the βC-peptide (25 nmol/min per mg) was of the same order of magnitude as with β-casein A^2 (38 nmol/min per mg) under the same phosphorylation conditions. It is possible that the replacement of the N-terminal cluster of 3 phosphoserines with aspartic residues could be responsible for the reduced affinity of the peptide substrate as compared to β-casein A^2. However, substantiation of this hypothesis requires the synthesis of new peptide(s) containing phosphoserine residues, which is not feasible by the traditional methods routinely employed for peptide synthesis.

Table 2 shows a number of other protein kinases which were not able to phosphorylate the βC-peptide at rates in any way comparable to those of reference substrates. No significant phosphorylation of this peptide by CK-2, cyclic AMP-dependent protein kinase, protein kinase C, phosphorylase kinase or protein kinase F_A could be detected.

From an operational point of view, a peptide substrate which is specific for just a given protein kinase is evidently useful, since it will allow for the direct and unambiguous monitoring of that individual enzyme

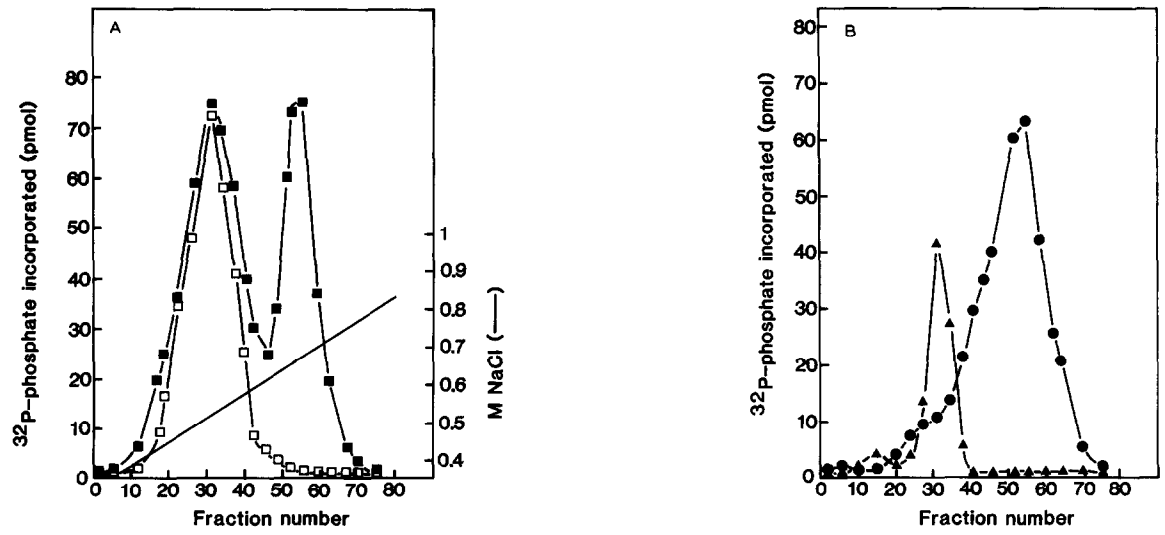

Fig.1. Purification of casein kinases by phosphocellulose chromatography. Aliquots of the column fractions (3 ml) were assayed for kinase activity toward (A) 2.5 mg/ml casein in the absence (■) or in the presence (□) of 5 μg/ml heparin and (B) toward 1.2 mM Ser-Glu-Glu-Glu-Glu-Glu (●) or 2.9 mM Asp-Asp-Asp-Glu-Glu-Ser-Ile-Thr-Arg-Arg (▲). Kinase activity is expressed as pmol of [^{32}P]phosphate incorporated in the substrate per assay and measured as described in section 2.

under conditions where a variety of other protein kinase activities are present. This is especially true for CK-1 since this enzyme is routinely assayed with casein, which is by no means a specific substrate for CK-1 alone: in fact, it is very readily phosphorylated by CK-2 and also significantly affected by a variety of other protein kinases, including phosphorylase kinase, protein kinase F_A, cyclic AMP-dependent protein kinase and several tyrosine protein kinases. The suitability of the βC-peptide for the specific detection of CK-1 has been confirmed here by its successful use in monitoring CK-1 activity during phosphocellulose chromatography, a very effective purification step for both classes of casein kinase (fig.1). Using casein as the substrate, CK-1 and CK-2 are equally detectable and the addition of specific inhibitors, such as heparin, is required in order to distinguish between the two enzymes. The phosphocellulose elution profiles of casein kinase activities from rat liver cytosol of fig.1, were obtained by using either whole casein or the synthetic peptides, Asp-Asp-Asp-Glu-Glu-Ser-Ile-Thr-Arg-Arg (βC-peptide) and Ser-Glu-Glu-Glu-Glu-Glu, as specific substrates for CK-1 and CK-2, respectively. As expected, the activity toward casein was resolved into two peaks (fig.1A): the first peak, eluting at 0.5 M NaCl is heparin-insensitive, a typical property of CK-1, while the more retarded peak eluting at 0.7 M NaCl is fully suppressed by heparin, denoting its type-2 nature. Fig.1B clearly shows that βC-peptide reveals a single peak of activity superimposed on the first heparin-insensitive casein kinase peak, while no significant βC-peptide kinase activity could be detected in the fractions containing the second casein kinase. The opposite is true if the assay is carried out with peptide, Ser-Glu-Glu-Glu-Glu-Glu, an excellent substrate for CK-2 [4] which is not affected by CK-1 (unpublished results). As also shown in fig.1B the only peak of activity in this latter case overlaps the more retarded, heparin-sensitive peak of CK-2. This experiment proves that the βC-peptide can be employed routinely for the specific detection and evaluation of CK-1, for which there was no suitable substrate available up to now. Therefore, it could represent a useful tool for investigating the physiological functions of CK-1, by making possible reliable estimates of its activity under a variety of conditions that might affect other protein kinase activities as well.

Acknowledgements: P.A. is a Research Associate and J.R.V. Research Director of the Belgian NFWO. This work was supported by the FGWO. The authors are grateful to R. Verbiest and R. Bollen for expert technical assistance.

REFERENCES

[1] Pinna, L.A., Meggio, F., Donella-Deana, A. and Brunati, A. (1985) Proc. 16th FEBS Congr., part A, pp. 155–163, VNU, Utrecht.
[2] Moore, A., Boulton, A.P., Heid, H.W., Jarasch, E.D. and Craig, R.K. (1985) Eur. J. Biochem. 152, 729–737.
[3] Hathaway, G.M. and Traugh, J.A. (1982) Curr. Top. Cell. Regul. 21, 101–127.
[4] Marin, O., Meggio, F., Marchiori, F., Borin, G. and Pinna, L.A. (1986) Eur. J. Biochem. 160, 239–244.
[5] Meggio, F., Marchiori, F., Borin, G., Chessa, G. and Pinna, L.A. (1984) J. Biol. Chem. 259, 14576–14579.
[6] Kuenzel, E.A., Mulligan, J.A., Sommercorn, J. and Krebs, E.G. (1987) J. Biol. Chem. 262, 9136–9140.
[7] Marchiori, F., Meggio, F., Marin, O., Borin, G., Calderan, A., Ruzzo, P. and Pinna, L.A. (1988) Biochim. Biophys. Acta 971, 332–338.
[8] Meggio, F., Perich, J.W., Johns, R.B. and Pinna, L.A. (1988) FEBS Lett. 237, 225–228.
[9] Meggio, F., Perich, J.W., Mejer, H.E., Hoffmann-Posorske, E., Lennon, D.P.W., Johns, R.B. and Pinna, L.A. (1989) Eur. J. Biochem., in press.
[10] Sommercorn, J., Mulligan, J.A., Lozeman, F.J. and Krebs, E.G. (1987) Proc. Natl. Acad. Sci. USA 84, 8834–8838.
[11] Ackerman, P. and Osheroff, N. (1989) J. Biol. Chem. 264, 11958–11965.
[12] Mercier, J.C., Maubois, J.L., Pozhanski, S. and Rebadeau-Dumams, B. (1968) Bull. Soc. Chim. Biol. 50, 521–530.
[13] Fischer, E.H. and Krebs, E.G. (1958) J. Biol. Chem. 231, 65–71.
[14] Tung, H.Y.L., Resink, T., Hemmings, B.A., Shenolikar, S. and Cohen, P. (1984) Eur. J. Biochem. 138, 635–641.
[15] Pickett-Gies, C.A. and Walsh, D.A. (1985) J. Biol. Chem. 260, 2046–2056.
[16] Sivaramakrishnan, S., Vandenheede, J.R. and Merlevede, W. (1983) Adv. Enz. Regul. 21, 321–330.
[17] Beavo, J., Bechtel, P. and Krebs, E.G. (1974) Methods Enzymol. 38, 299–308.
[18] Parker, P.J., Stabel, S. and Waterfield, M.D. (1984) EMBO J. 3, 953–959.
[19] Meggio, F., Deana, A.D. and Pinna, L.A. (1981) J. Biol. Chem. 256, 11958–11961.
[20] Glass, D.B., Masaracchia, R.A., Feramisco, J.R. and Kemp, B.E. (1978) Anal. Biochem. 87, 566–575.
[21] Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1971) J. Biol. Chem. 246, 1986–1995.
[22] Agostinis, P., Goris, J., Pinna, L.A. and Merlevede, W. (1987) Biochem. J. 248, 785–789.
[23] Vandenheede, J.R., Yang, S.-D., Goris, J. and Merlevede, W. (1980) J. Biol. Chem. 255, 11768–11774.
[24] Donella-Deana, A., Meggio, F. and Pinna, L.A. (1979) Biochem. J. 179, 693–696.
[25] Tuazon, P.T., Bingham, E. and Traugh, J.A. (1979) Eur. J. Biochem. 94, 497–504.
[26] Meggio, F., Donella-Deana, A. and Pinna, L.A. (1979) FEBS Lett. 106, 76–80.
[27] Flotow, H. and Roach, P.J. (1989) J. Biol. Chem. 264, 9126–9128.
[28] Grasser, F.A., Scheidtmann, K.H., Tuazon, P.T., Traugh, J.A. and Walter, G. (1988) Virology 165, 13–22.
[29] Agostinis, P., Vandenheede, J.R., Goris, J., Meggio, F., Pinna, L.A. and Merlevede, W. (1987) FEBS Lett. 224, 385–390.