ELSEVIER

Journal of Chromatography A, 712 (1995) 169–175

JOURNAL OF CHROMATOGRAPHY A

Liquid chromatographic–mass spectrometric studies on the enzymatic degradation of β-endorphin by endothelial cells

M. Brudel, U. Kertscher, D. Schröder, M.F. Melzig, B. Mehlis*

Institute of Molecular Pharmacology, A. Kowalke Str. 4, D-10315 Berlin, Germany

Abstract

An on-line HPLC–mass spectrometric procedure with an electrospray atmospheric pressure ionization (ESI-API) ion source was developed to identify the enzymatic degradation products (peptides) generated by incubation of human β-endorphin (hβE) with cultured aortic endothelial cells. The samples from the complex incubation mixture were prepurified and enriched using a small reversed-phase (RP) perfusion precolumn. Flow switching was applied to transfer the peptides from this precolumn to the analytical RP column of 2 or 0.32 mm I.D. and to separate them by gradient elution. The peptides were detected by means of an on-line coupled triple quadrupole mass spectrometer (TSQ 700) with an ESI-API ion source operated in the positive ion mode. This MS system behaves as a concentration sensitive detector at flow-rates from 5 to 150 μl/min. MS–MS experiments supported the unambiguous assignment of the peptide structures. Thus most of the peptide fractions were identified and the region 16-17-18 (-L-F-K-) of hβE was found to be primarily attacked by the enzymes of the endothelial cells.

1. Introduction

The neuropeptide β-endorphin (βE) is known as one of the most active endogenous opioides and is involved in many physiological and pathophysiological processes. It is generated from its precursor pro-opiomelanocortin concomitantly with other neuropeptides by selective proteolytic hydrolysis in the brain and pituitary [1] resulting in a peptide with 31 amino acid residues, i.e. human βE(1–31):

$Y^{1}GGFMTSEKS^{10}QTPLVTLFKN^{20}AIIKNAY-KKGE^{31}$.

βE is also distributed in many peripheral tissues [2,3].

Secondary peptidolytic processing of βE has been reported to proceed in the pituitary and brain where C-terminal attack results in βE(1–27) and βE(1–26) as the naturally occurring fragments [4,5]. Another cleavage site is the Leu^{17}-Phe^{18} bond of βE resulting in the biologically active fragment βE(1–17), referred to as γ-endorphin [6,7].

The endothelial cells of the blood vessels are able to process biologically active peptides. This has been reported for Leu-enkephalin [8,9], angiotensin I [10], endothelin [11], bradykinin [12] and natriuretic peptide [13]. However, no reference to the fate of βE in contact with endothelial cells is available.

It was our aim to gain information on this process because exogenous βE, applied in pharmacological experiments, has to pass from the circulation through the continuous monolayer of

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00564-1

endothelial cells at the inner surface of the blood vessels to its target cells and may be converted by these cells into fragments of changed bioactivity.

Liquid chromatography–mass spectrometry with electrospray ionization is an established method for the identification of peptide molecules. It is also very suitable for the identification of peptide fragments in tryptic or similar peptide digests [14,15]. However, when the peptides have to be identified in a biological matrix where a complex of peptidases of unknown specificity generates a number of fragments, difficulties may arise. The processing of βE, a 31 amino-acid residue peptide, in contact with living cells can serve as a model in this respect. In addition it was our aim to analyze the fractions containing low peptide concentrations that were detected by the UV detector but were below the limit of detection of the mass spectrometer. Therefore, the HPLC–MS system was combined with a flow-switching setup with a small precolumn effecting both fast on-line prepurification and enrichment. Thus we were able to analyze the samples obtained from our pharmacological experiments containing nanomolar peptide concentrations.

2. Experimental

2.1. Materials

Human β-endorphin, hβE = hβE(1–31), and its partial sequences were supplied by Bachem (Heidelberg, Germany). A small amount ($< 5\%$) of oxidized peptide (Met-sulfoxide) was present in these preparations after dissolution. Acetonitrile (HPLC ultra gradient grade) was from Baker (Gross Gerau, Germany). Trifluoroacetic acid (TFA) spectral quality (Uvasol) and all other chemicals were from Merck (Darmstadt, Germany). Deionized water was obtained from a combined Milli-RO/Milli-Q plus water system (Millipore, Eschborn, Germany).

2.2. Incubation of cells with hβE

Endothelial cells from calf aorta (line BKEz-7) were grown to form a confluent monolayer as previously described [16]. The culture medium was removed and the cell layer (ca. $3 \cdot 10^6$ cells) washed two times with an isotonic solution (NaCl 8 g, KCl 0.2 g, $Na_2HPO_4 + 12\ H_2O$ 2.9 g, KH_2PO_4 0.2 g in 1000 ml deionized H_2O). A solution of hβE (1.2 ml, max. concentration 20 μM) in this isotonic solution was added to cover the cell layer completely and incubated at 37°C with gentle shaking for a maximum of 2 h. Samples of the supernatant were collected at time intervals, taking care not to damage the cells, acidified with TFA (final concentration 1%, pH ca. 1.4), and then centrifuged at 9500 *g* for 5 min at 4°C to remove cell fragments.

2.3. Mass spectrometry

The mass spectrometric equipment was a triple quadrupole mass spectrometer (Finnigan MAT TSQ 700, Bremen, Germany) with an electrospray (API) ion source (Finnigan MAT) operating in the positive mode with a capillary temperature of 200°C, a high voltage of 4.5 kV, nitrogen as auxiliary and sheath gas and without sheath liquid. The off-line mass spectra were obtained by syringe infusion of the sample in methanol–water (1:1) containing 1% acetic acid with a flow of 3 to 5 μl/min (except for the flow experiments) and with a sheath gas flow corresponding to $2.1 \cdot 10^5$ Pa without an auxiliary gas flow. In the on-line mode the API ion source was operated at a flow of 5–100 μl/min (HPLC eluent) depending on the column used and with a sheath and auxiliary gas flow corresponding to $2.8 \cdot 10^5$ Pa and 235 ml/min, respectively.

Off-line MS–MS experiments were performed with the respective peptide fractions by using a collision gas pressure of 1–3 mTorr, offset voltage of −20 to −30 eV and multiplier voltage of 1.6–1.8 kV.

2.4. Chromatographic system

The system used for the on-line LC–MS studies consisted of an Applied Biosystems dual-syringe pump, type 140B and UV detector type 785A equipped with a micro flow cell (light path 8 mm, quartz capillary, type UZ, LC Packings/ICT, Bad Homburg, Germany). Separations

were carried out using columns of Vydac 300 Å, C_{18}, 5 μm, 150 × 2 mm I.D. (Promochem, Wesel, Germany) and Vydac 300 Å, C_{18}, 5 μm, 150 × 0.32 mm I.D. (LC-Packings/ICT). The flow lines applied for these two columns were similar to those described earlier [17] and included post- and precolumn splitting for the 2 mm and 0.3 mm columns, respectively. In general a split ratio (X/Y) of 9:1 for the 2 mm column and 1:19 for the 0.3 mm column was applied (X = flow to the MS system). When peptide fractions had to be analyzed off-line, the split ratio at the 2 mm column was reversed (X/Y = 1:9, main flow collected). Samples were injected using a trace enrichment setup [18] with a two-position 10-port valve with electric actuator (Valco, type WE C10 WK). The sample loop (PEEK tubing) was filled with the valve in position A, while with the valve in position B the sample was delivered to the precolumn and then flushed with two volumes of water with the aid of an auxiliary pump (LC-6A, Shimadzu, Duisburg, Germany). The precolumns were dry filled with RP perfusion stationary phase POROS 10 R2, 10 μm (PerSeptive Biosystems, Freiburg, Germany). A 20 × 2 mm I.D. precolumn (Upchurch cat. no. C.130B) was used for the 2 mm analytical column and a 10 × 1 mm I.D. precolumn for the 0.3 mm column. Gradient elution was performed with the valve in position A with a mobile phase consisting of A: acetonitrile–water (1:99) containing 0.05% TFA and B: acetonitrile–water (80:20) containing 0.05% TFA. The gradient was run from 15% B to 47% B in 20 min and then in 5 min to 100% B.

Edman sequencing of peptide fractions was performed with a protein sequencer (type 476A, PE/Applied Biosystems, Weiterstadt, Germany).

3. Results and discussion

The mass chromatograms (overlay) in Fig. 1 show that hβE is degraded by the enzymes of the endothelial cells into various peptide fragments. Some of the chromatographic peaks (e.g. trace A, no. 11/12 and 14/16; trace B, no. 5/6) detected by the mass spectrometer strongly overlap and were not discriminated by the UV detector. Comparison of traces A and B also demonstrates the advantage of the microcolumn (trace A) with a low amount of peptide in the sample. In our experiments a two- to threefold increase in the MS signal intensity was obtained for the microcolumn over that obtained with the 2 mm I.D. column. However, when a sufficient amount of peptide was available, the 2 mm I.D. column was preferred because of its ruggedness. There was also no significant decrease in MS signal intensity when the flow-rate to the MS was increased from 5 μl/min to 150 μl/min (Fig. 2). As can be seen in this figure, the MS system with the ESI-API ion source applied in this study behaves as a concentration sensitive detector over the applied range of flow-rates with a flow optimum indicated around 50 μl/min.

Identification of peptides in biological samples is often difficult because of the low peptide concentrations and matrix effects. The setup for prepurification used in this study significantly improved the MS measurements. Large sample volumes ($\leq$2 ml) were applied with high flow-rates (ca. 5 precolumn volumes/min) using RP perfusion stationary phase [15,19]. The dynamic capacity of the 2 mm I.D. precolumn was determined to be ca. 0.1 μmol of peptide (concentration 0.1 μmol/ml). This method can be applied at high salt concentrations (e.g. 0.9% NaCl) provided the samples do not contain high concentrations of proteins competing with the peptides for the stationary phase.

In Table 1 the assignment of the chromatographic peaks (Fig. 1) to the respective hβE fragments is summarized. Most of the fragments were assigned by their double and triple charged ions in the ESI–MS spectra. The structural assignment by mass determination alone (at unit mass resolution) is often not feasible when peptide fragments of this size have to be analyzed. One example are the sequences hβE(19–31) and hβE(7–19), both with M_r 1477.7. Therefore, off-line MS–MS and micro Edman sequencing experiments were performed, where necessary, to confirm the assignment as shown in Table 1. Thus most of the fragments have been unambiguously identified, except the minor peaks 3, 4, 8 and 12. Peak no. 3, which is proposed to be

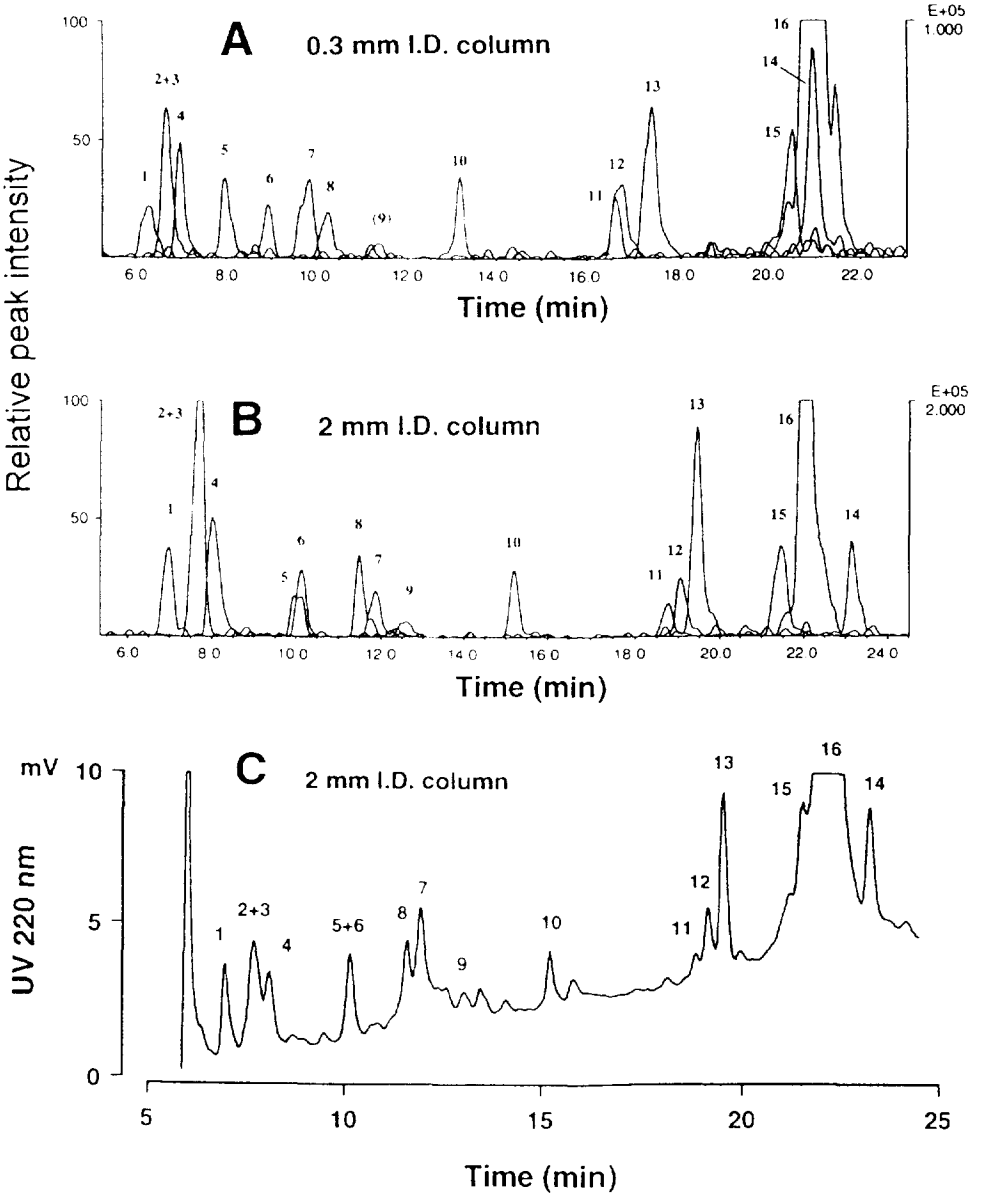

Fig. 1. Mass chromatograms (traces A and B) of the peptide fragments obtained after incubation of hβE for 2 h with cultured endothelial cells. The individual mass traces of the $[M + 2H]^{2+}$ or $[M + 3H]^{3+}$ ions are superimposed to give the overlap traces. Trace A is the mass chromatogram of the degradation mixture (initial concentration of hβE 0.4 μM) with the 0.3 mm I.D. column setup while trace B was of an initial 2 μM concentration of hβE and run with the 2 mm I.D. column setup. The concentrations of the fragments in trace A and B were ca. 0.02–0.1 μM and 0.1–0.5 μM respectively. In trace C the UV absorption corresponding to trace B is depicted. For the structural assignment of the fragments see Table 1. Peaks no. 16 and 15 are hβE and its Met-sulfoxide, respectively. Peaks no. 4 and 12 could not be assigned.

hβE(19–30), was detected only when a very shallow gradient was applied (data not shown).

The peptidases of the endothelial cells degrade about 60% of the hβE applied in the incubation mixture within 2 h under the conditions used here for the incubation (Fig. 3, insert). During

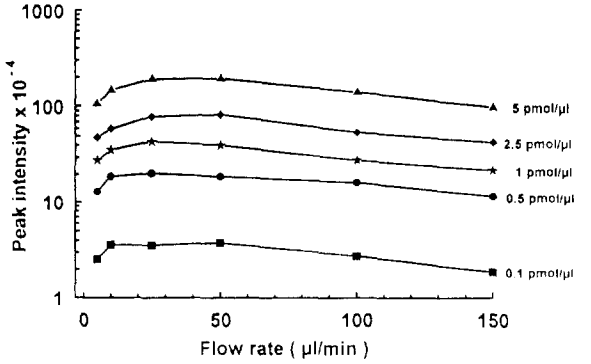

Fig. 2. ESI-API–MS signal intensity at different concentrations and flow-rates. The peptide samples (5 μl) of 0.1–5 μM solutions of hβE(1–27) in the solvent acetonitrile–water (25:75) containing 0.05% TFA were injected in the flow injection mode into the same solvent. Data points are mean values from two independent sets of experiments with each concentration injected in duplicate (R.S.D. $\leq$10%). Spray parameters (sheath gas pressure, auxiliary gas flow, capillary temperature) were optimized at each flow-rate to get a stable spray.

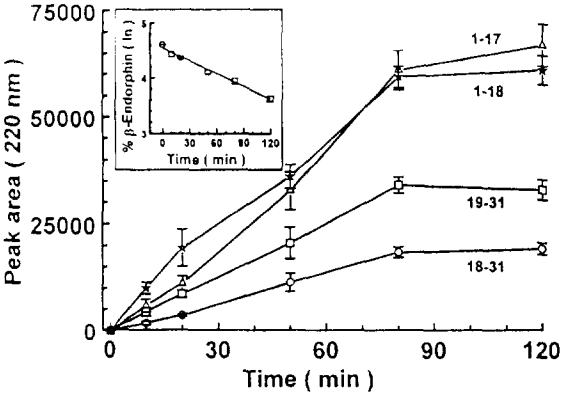

Fig. 3. Time course of the formation of four main degradation products of hβE in the incubation mixture with the endothelial cells. hβE sequences: (△) 1–17, (☆) 1–18, (□) 19–31, (○) 18–31. The half-life of hβE (cf. insert) in presence of the endothelial cells was determined to be 82 min ($n = 5$).

this period the fragments hβE(1–17) and hβE(1–18) show the highest concentration. The complementary fragments hβE(18–31) and hβE(19–31) also increase with time (Fig. 3) but do not reach the level of the former fragments, probably because of their rapid degradation by amino- and carboxypeptidases, resulting in the intense peak no. 2 + 3, hβE(20–31) + hβE(19–30). The processing of hβE by the endothelial cells may proceed as shown in Fig. 4 with the region 16-17-18 (-L-F-K-) of hβE as the primary site of enzymatic attack. Thus, the fragment

Table 1
Fragments of human β-endorphin formed by the enzymes of the endothelial cells

Peptide sequence	β-Endorphin fragment	Peak No	M_r(calc.)[b]	m/z[c]	
				$[M + 2H]^{2+}$	$[M + 3H]^{3+}$
YGGFMTSEKSQTP	1–13	7	1432.6	717.4	478.7
GGFMTSEKSQTP	2–13	8	1269.4	635.7	424.2
YGGFMTSEKSQTPLVT	1–16	10	1746.0	873.7	582.6
YGGFMTSEKSQTPLVTL	1–17	13	1859.1	930.8	620.9
GGFMTSEKSQTPLVTL	2–17	11	1696.0	848.9	–[d]
YGGFMTSEKSQTPLVTLF	1–18	14	2006.3	1003.8	670.0
LFKNAIIKNAYKKGE	17–31	9	1737.1	869.3	579.8
FKNAIIKNAYKKGE	18–31	6	1623.9	813.0	542.6
KNAIIKNAYKKGE	19–31	1	1476.8	739.7	493.3
KNAIIKNAYKKG	19–30	3	1347.6	675.2	450.2
NAIIKNAYKKGE	20–31	2	1348.6	675.4	450.6

[a] Peak numbers refer to Fig. 1. Peak no. 16 (hβE), M_r(calc.) 3465.2 shows m/z 1733.5 and 1156.1 for the double and triple charged ion, respectively.
[b] Calculated relative molecular mass (average mass).
[c] m/z values of the ions observed in the ES–MS spectra.
[d] $[M + 3H]^{3+}$ ion not observed, found: $[M + H]^{+}$, m/z 1696.5.

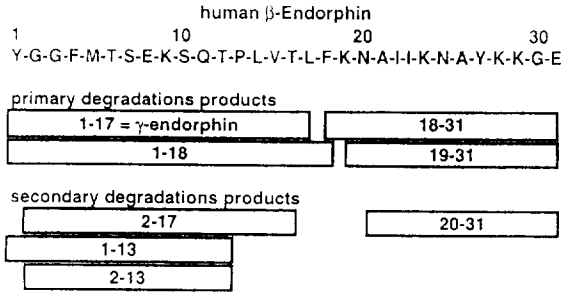

Fig. 4. Degradation pattern of hβE produced by the enzymes of the endothelial cells. The site of the primary enzymatic attack is the region 16-17-18. Secondary fragments (only examples shown, see also Table 1) may be generated by amino- and carboxypeptidases from the primary fragments.

hβE(1–17), γ-endorphin, identified in some tissues [6,7], is also generated by the enzymes of the endothelial cells from hβE. It can not be deduced from these results that enzymes exclusively located at the surface of the endothelial cells are involved. Experiments on the nature and localization of these enzymes are in progress and will be published elsewhere. Apparently, two enzymes are responsible for the splitting in the L-F-K region as shown by the detection of hβE(1–18). The sequences hβE(1–26) and hβE(1–27), also known as naturally occurring fragments [4,5], were not detected and may only be produced (if at all) in very low amounts in this system. Whether the fragment hβE(1–13), observed in our experiments, is generated by selective endopeptidase attack or through carboxypeptidase degradation of the larger N-terminal fragments remains to be established.

4. Conclusions

The experimental procedure described in this paper enabled us to structurally assign most of the fragments of the 31 amino acid peptide hβE which were produced in the incubation of hβE with the endothelial cells. We think that this procedure can be generally applied to the study of enzymatic processing of peptides as a result of their contact with cells.

It is concluded that the on-line HPLC–MS approach gives a valuable fast overview on the possible peptide fragments. However, if peptides of nearly equal masses have to be considered, unambiguous structural assignment requires additional information. This we obtained from off-line MS–MS experiments which were carried out with fractions collected from the gradient elution. In future it would be desirable to perform the daughter ion experiments on-line as proposed recently by Cary et al. [20].

The applied enrichment procedure using a perfusion stationary phase is fast and reliable. It allows the analysis of low micromolar peptide concentrations in the cell incubation experiments and identification of fragments at nanomolar concentrations. However, further improvements will be necessary for the processing of peptides larger than hβE and at nanomolar concentrations to be studied.

Acknowledgements

We thank H. Lerch and D. Runald for their skilful technical assistance and Dr. H. Berger for helpful discussions. The authors acknowledge the financial support of the Bundesministerium für Forschung und Technologie (BMFT), grant No. 07 NBL 07-6 TP 06.

References

[1] Y.P. Loh, Biochem. Pharmacol., 44 (1992) 843.
[2] C.R. DeBold, W.E. Nicholson and D.N. Orth, Endocrinology, 66 (1988) 2648.
[3] A.I. Smith and J.W. Funder, Endocrine Rev., 9 (1988) 159.
[4] W.R. Millington and D.L. Smith, J. Neurochem., 57 (1991) 775.
[5] M.D. Hirsch and W.R. Millington, Brain Res., 550 (1991) 61.
[6] D.M. Dorsa, M.B. Chapman and D.G. Baslin, Peptides, 3 (1982) 455.
[7] J.L.M. Lebouille, R.W. Hendriks, N.M. Soeter and J.P.H. Burbach, J. Neurochem., 52 (1989) 1714.
[8] G. Heder, M. Melzig, W.E. Siems, Pharmazie, 47 (1992) 226.
[9] S.E. Thompson and K.L. Audus, Peptides, 15 (1994) 109.

[10] R. Auerbach, L. Alby, J. Grieves, J. Joseph, C. Lingren, L.W. Morrissey, Y.A. Sidky, M. Tu and S.L. Watts, Proc. Natl. Acad. Sci. USA, 79 (1982) 7891.
[11] T.J. Opgenorth, J.R. Wu-Wong and K. Shiosaki, FASEB J., 6 (1992) 2653.
[12] K. Graf, M. Gräfe, C. Bossaller, J. Niehus, K.-D. Schulz, W. Auch-Schwelk and E. Fleck, Eur. J. Clin. Chem. Clin. Biochem., 31 (1993) 267.
[13] S.J. Frost and P.A. Whitson, J. Cell. Biochem., 52 (1993) 227.
[14] T.R. Covey, E. Huang and J.D. Henion, Anal. Chem., 63 (1991) 1193.
[15] D.B. Kassel, B. Shushan, T. Sakuma and J.P. Salzmann, Anal. Chem., 66 (1994) 236.
[16] M. Brudel, U. Kertscher, H. Berger and B. Mehlis, J. Chromatogr. A, 661 (1994) 55.
[17] W. Halle, W.-E. Siems, K.D. Jentzsch, E. Teuscher and E. Göres, Pharmazie, 39 (1984) 77.
[18] VICI AG, Valco Europa, Schenkon, Switzerland, Catalogue, 1993, p. 15.
[19] S.P. Fulton, N.B. Afeyan, N.F. Gordon and F.E. Regnier, J. Chromatogr., 547 (1991) 452.
[20] A.M. Cary, I. Jardine, M. Wakefield, A. Land and C. Campbell, presented at the *11th Montreux Symposium on Liquid Chromatography–Mass Spectrometry, Montreux, 9–11 November 1994.*