Molecular Diversity **3:** 29–41, 1997.

Composition and purity of combinatorial aryl ether collections analyzed by electrospray mass spectrometry

Wolfgang J. Haap[a], Jörg W. Metzger[b], Christoph Kempter[b] & Günther Jung[a,*]
[a] *Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, D-72076 Tübingen, Germany*
[b] *Institute of Sanitary Engineering, Water Quality and Solid Waste Management, University of Stuttgart, Bandtäle 2, D-70569 Stuttgart, Germany*

Received 14 January 1997; Accepted 17 April 1997

Key words: aryl ether library, combinatorial chemistry, electrospray mass spectrometry, HPLC-MS, molecule libraries, tandem MS

Summary

Electrospray mass spectrometry (ESI-MS), tandem mass spectrometry and on-line RP-HPLC-ESI-MS were used to evaluate the composition and purity of three different aryl ether mixtures consisting of 10 and 45 aryl ethers synthesized on solid support by Williamson etherification. The libraries feature two potential pharmacophores connected with three different spacers and serve as models for a detailed component analysis. Individual members of the library and by-products were identified rapidly and conveniently by product ion scans. Compound collections obtained by two different synthetic methods, the split/combine approach and the premix method, showed different mass distributions in the ESI-MS spectra. Some components were not detected in direct ESI-MS measurements, but were found by MS/MS experiments. Precursor ion and constant neutral loss scans allowed the identification of components with common structural features.

Introduction

The synthesis of small molecule libraries using combinatorial chemistry methods is playing an increasingly significant role in accelerating the drug discovery process [1–4]. Since peptide libraries tend to have limitations as therapeutic agents (poor oral bioavailability, proteolytic instability), most research efforts are concentrated in transferring solution phase organic reactions onto the solid support in order to synthesize non-oligomeric small molecules [5–8]. Many solid phase syntheses of small molecule libraries have been reported during the last few years. For both quality control and the determination of composition and purity, most of these libraries were fingerprinted simply by HPLC [9,10] or characterized by MS [11]. Only a few reports show the application of combining a separation method with MS, e.g. CE-MS [12]. Often the mass spectra of small molecules contain fragment ions, so that the interpretation of the mass spectrum of a library is difficult [13]. HPLC, GC and CE analyses alone are inadequate in the case of more complex mixtures and coelution of some components is the rule for libraries containing more than 50 expected products and unknown impurities.

In order to demonstrate the potential of ESI-MS, MS/MS, HPLC-MS and HPLC-MS/MS for the determination of the composition and purity of 'small molecule libraries' [13–15], three different aryl ether libraries [16–19] with 10 and 45 components were synthesized by Williamson etherification. The libraries contain two potential pharmacophores, an amino acid and a phenol derivative, which are connected with three different spacers. As reported by Krchňák et al. [19], streptavidin ligands were found from this class of compounds.

Compared to other analytical methods, modern mass spectrometric techniques allow very fast and con-

* To whom correspondence should be addressed.

venient identification of the constituents and the by-products of libraries.

Materials and Methods

General
Solvents and reagents were obtained from commercial suppliers and used without further purification. Multiple solid phase syntheses were performed on polystyrene/1% divinylbenzene A RAM resin (200–400 mesh, capacity 0.63 mmol/g). All solid phase reactions were performed in syringes equipped with a frit.

Library synthesis: General method
Three different libraries were prepared (Figure 1): (i) a library containing 10 aryl ethers (*Fa1-5, Ka1-5*) using the 'premix' method [20,21]; (ii) a library containing 45 aryl ethers using the 'split and combine' approach [22]; and (iii) a library containing 45 aryl ethers using the 'premix' method.

Library i Fmoc protected polystyrene A RAM resin (capacity 0.63 mmol/g; 100 mg, 0.063 mmol) was treated with piperidine/DMF (1:1, 2 ml) at room temperature for 0.5 h, drained and washed with DMF (6×). A solution of Fmoc-Phe-OH in DMF (0.5 M, 0.063 ml, 0.5 equiv), Fmoc-Lys(Boc)-OH in DMF (0.5 M, 0.063 ml, 0.5 equiv) and 1-hydroxybenzotriazol (HOBt) (0.063 mmol, 1 equiv) was diluted with DMF (1.5 ml) and added to the resin. *N,N′*-diisopropylcarbodiimide (DIC) (1.2 equiv) was added and the mixture was agitated for 3 h at room temperature. The resin was washed with DMF (5×). The coupling of the amino acids was repeated and the Fmoc group was cleaved as described above. The resin was acylated with 2-bromoacetic acid (6.3 mmol, 10 equiv) by in situ activation with DIC (12 equiv) for 3 h at room temperature. A mixture of five different phenols (Scheme 1) (0.2 equiv each, in total 1 equiv) in dry DMSO (1.5 ml) was treated with sodium ethanolate (1 equiv) for 0.5 h and added to the resin. After 3 h at room temperature, a second coupling of the phenolates was performed using a large excess of the phenolates (in total 30 equiv) and sodium ethanolate (30 equiv). After extensive washings the aryl ether mixture was cleaved from the solid support with trifluoroacetic acid/dichloromethane (1:1) within 3 h. The filtrate was evaporated and dissolved in methanol, and the crude product was analysed as described below.

Library ii Fmoc protected polystyrene A RAM resin (capacity 0.63 mmol/g; 300 mg, 0.189 mmol) was divided into three equal portions. The Fmoc group was removed as described above. Solutions of protected amino acids and HOBt, Fmoc-Lys(Boc)-OH/HOBt (0.5 M amino acid, 0.5 M HOBt; 1 ml, 8 equiv), Fmoc-Arg(Pmc)-OH/HOBt (0.5 M, 1 ml, 8 equiv), Fmoc-Phe-OH/HOBt (0.5 M, 1 ml, 8 equiv) in DMF and DIC (0.63 mmol, 10 equiv) were added to the individual resin portions and agitated at room temperature for 3 h. Each reaction mixture was filtered individually, then recombined and washed with DMF (8×). After removal of the Fmoc group using piperidine in DMF and washing with DMF (8×), DCM (8×) and methanol (6×), the pooled resin was dried to a constant weight and divided into three equal portions. Individual acylations were performed with 2-bromoacetic acid (0.63 mmol, 10 equiv), 6-bromohexanoic acid (0.63 mmol, 10 equiv) and 4-(bromomethyl) benzoic acid (0.63 mmol, 10 equiv) in DMF (1 ml) using DIC (0.76 mmol, 12 equiv) for activation. The individual reaction mixtures were agitated for 3 h at room temperature, filtered individually, recombined and washed with DMF (4×) and DCM (4×). After drying to a constant weight, the resin was split into five equal portions. Each phenol derivative (see Scheme 1) (1.89 mmol, 30 equiv) was dissolved in dry DMSO (1 ml) and treated with sodium ethanolate (1.89 mmol, 30 equiv) for 20 min. The sodium phenolates were individually added to the resin and the mixtures were agitated for 3 h at room temperature. Each mixture was filtered individually, and the resin portions were recombined and washed with DMF (6×), methanol (4×) and DCM (4×). The resin was dried and treated with trifluoroacetic acid/DCM (1:1) for 3 h. The mixture was evaporated and samples dissolved in methanol were analyzed by ESI-MS.

Library iii The 'premix library' containing 45 aryl ethers was synthesized as described for library ii, except for the coupling of the phenolates. An equimolar mixture of all phenols (in total 1 equiv) and sodium ethanolate (1 equiv) in dry DMSO (3 ml) was added to the resin (capacity 0.63 mmol/g; 300 mg, 0.189 mmol) after 20 min of preincubation. The mixture was agitated at room temperature for 3 h. The resin was washed with DMSO (5×) and the nucleophilic displacement was repeated with a mixture of all phenols (in total 30 equiv) and sodium ethanolate (30 equiv) in DMSO (3 ml) at room temperature for 3 h. The resin was washed with DMF (6×), methanol (4×) and DCM (4×). The resin was dried and treated with trifluo-

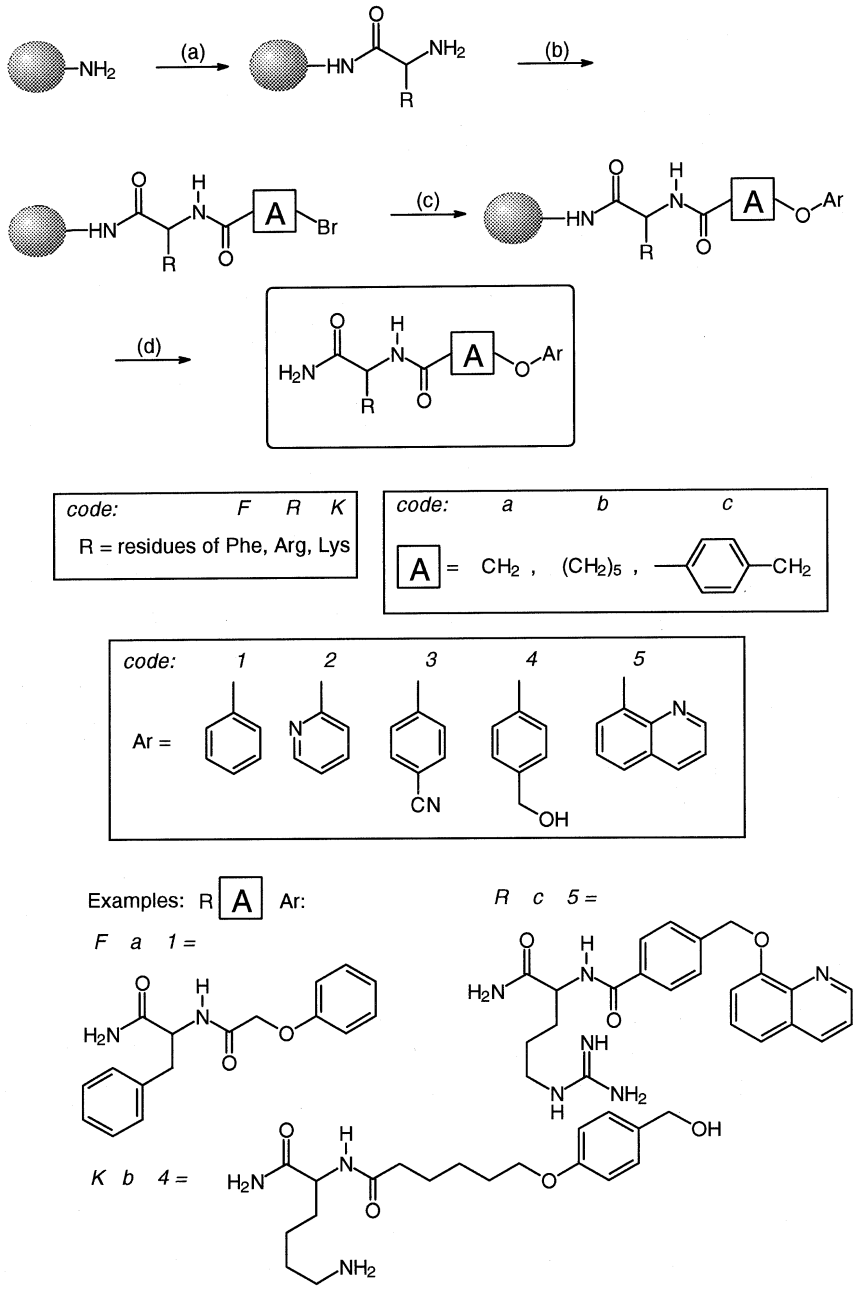

Figure 1. Solid-phase synthesis of aryl ether libraries and some examples of the synthesized compounds. (a) 0.5 M amino acid/HOBt in DMF, DIC, 3 h, rt; (b) 10 equiv bromo carboxylic acid in DMF, DIC, 3 h, rt; (c) 1–30 equiv phenol derivatives (see text), 1–30 equiv sodium ethanolate, DMSO, 1–2 × 3 h, rt; (d) trifluoroacetic acid:dichloromethane (1:1), 3 h, rt. Shaded circle = polystyrene A RAM resin (200–400 mesh, capacity 0.63 mmol/g).

roacetic acid/DCM (1:1) for 3 h. After evaporation the residue was dissolved in methanol and analyzed by ESI-MS.

Mass spectrometry

Positive ion ESI-MS, MS/MS, HPLC-MS and HPLC-MS/MS data were recorded on a Perkin Elmer Sciex API III (Toronto, ON, Canada) mass spectrometer

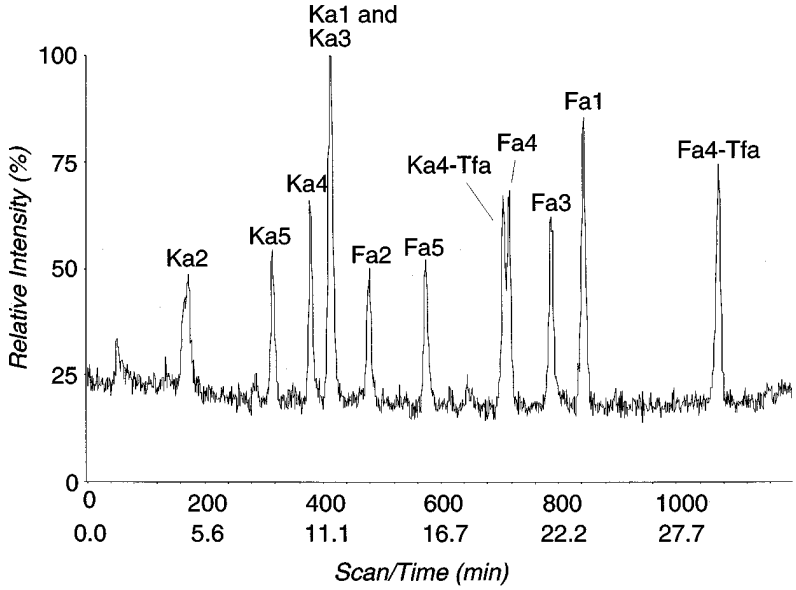

Figure 2. Total ion current chromatogram (TIC) of the crude 2 × 5 aryl ether library. For abbreviations see Figure 1; Tfa = *O*-trifluoroacetylated aryl ether.

equipped with a nebulizer-assisted electrospray source. ESI mass spectra were acquired at unit resolution scanning with a step size of 0.5 u and a dwell time of 1 ms. Five to ten spectra were summed. The potential of the spray needle was held at +4.8 kV and spectra were recorded at orifice voltages of +70 to +100 V.

Tandem mass spectrometry (product ion, precursor ion and neutral loss scans) was performed using collision-induced dissociation (CID) with argon and collision energies ranging from 30 to 60 eV.

RP-HPLC chromatograms were obtained on an Applied Biosystems 140 A Delivery System (Weiterstadt, Germany) equipped with a Gromsil ODS 5-ST 5 μm C_{18} column (Grom, Herrenberg, Germany) (column dimension 100 × 2 mm, flow rate 200 μl/min, split of 40 μl/min into ESI source) and a Linear UVis 204 detector (Reno, Nevada, U.S.A.). UV absorbance (detection at 214 nm) was detected simultaneously with the MS signal and registered with a Shimadzu Chromatopac data processor C-R1 B (Duisburg, Germany). The UV trace was also acquired directly onto the data system of the mass spectrometer, allowing a direct comparison of UV trace and total ion current chromatogram (TIC). Binary linear gradients were used (solvent A = water/0.1% trifluoroacetic acid, solvent B = acetonitrile/0.1% trifluoroacetic acid).

Table 1. Composition of the crude 2 × 5 aryl ether library

Compound	Peak areas (%)	
	HPLC[a] (214 nm)	TIC[b]
Ka1	13.3 (Ka3)	16.6 (Ka3)
Ka2	5.7	3.3
Ka3	13.3 (Ka1)	16.6 (Ka1)
Ka4	6.7	9.6
Ka5	6.9	7.1
Ka4-Tfa[c]	3.2	7.2
Fa1	11.1	14.0
Fa2	13.5	6.0
Fa3	10.6	8.7
Fa4	5.7	7.7
Fa5	9.9	6.9
Fa4-Tfa	6.8	11.5

[a] Peak areas in the UV trace.
[b] Peak areas in the TIC.
[c] –Tfa = trifluoroacetylated hydroxymethylphenyl ethers.

Results and Discussion

MS analysis of a (2 × 5) library

We first investigated a 2 × 5 component library (*Fa1-5* and *Ka1-5*) by RP-HPLC-ESI-MS. The TIC of the 2 × 5 library shows 11 baseline separated peaks (Figure 2). The corresponding masses indicated that all

expected $[M + H]^+$-ions of the library were present and that compounds *Ka1* and *Ka3* were coeluted (Figure 3). In addition, two by-products were revealed which could be identified by tandem-MS (vide infra) as the *O*-trifluoroacetylated hydroxymethylphenyl ethers *Ka4-Tfa* and *Fa4-Tfa*. The peak areas (UV detection at 214 nm) of the detected components indicated a purity greater than 83%. The concentration of the by-products which were formed during cleavage was determined to be less than 10% in this library (Table 1). In contrast, the concentration of by-products determined via the TIC peak areas was higher than that determined from the UV trace (Table 1). Such deviations were also observed for peptides and peptide libraries [13] and result from the different ionization yields of the individual components. Additional by-products, e.g. formed by incomplete coupling or deprotection steps, were not found.

2D analysis of HPLC-MS data

Characteristic fragment ions of single compounds, which are produced in the transport region of the ion source, or coeluting compounds can be identified in a 2D contour plot, in which m/z is plotted versus the retention time (Figure 3). Since no fragment ion occurred at *m/z* 280 in the product ion spectrum of *Ka3* ($[M + H]^+$ at *m/z* 305, Table 2), *Ka1* ($[M + H]^+$ at *m/z* 280) and *Ka3* must be coeluted (Figure 3).

On the other hand, the correlation of by-products with their corresponding aryl ethers is possible by following the horizontal lines in Figure 3. The occurrence of isobaric fragment ions of *Fa4* at *m/z* 329 (Table 2) and a so far unidentified impurity suggested that this by-product is trifluoroacetylated *Fa4* (*Fa4-Tfa*; $[M + H]^+$ at *m/z* 425, Table 2). This result was confirmed by on-line tandem MS (vide infra). Similar assignments of *Ka4* ($[M + H]^+$ at *m/z* 310, see Table 2) and *Ka4-Tfa* ($[M + H]^+$ at *m/z* 406, Table 2) can be made by comparing the fragmentation patterns shown in Figure 4.

Tandem mass spectrometry

Identification of constituents of the library and by-products was performed using on-line tandem mass spectrometry. Three different types of scans are commonly used: product ion scans, precursor ion scans and constant neutral loss scans [13].

We analyzed the 2×5 library by on-line HPLC-MS/MS. Product ion scans of the eluting compounds were recorded. The *m/z* value of the first analyzer (selection of the precursor ion) was switched depending on the *m/z* value of the protonated molecule ion of the presently eluting compound.

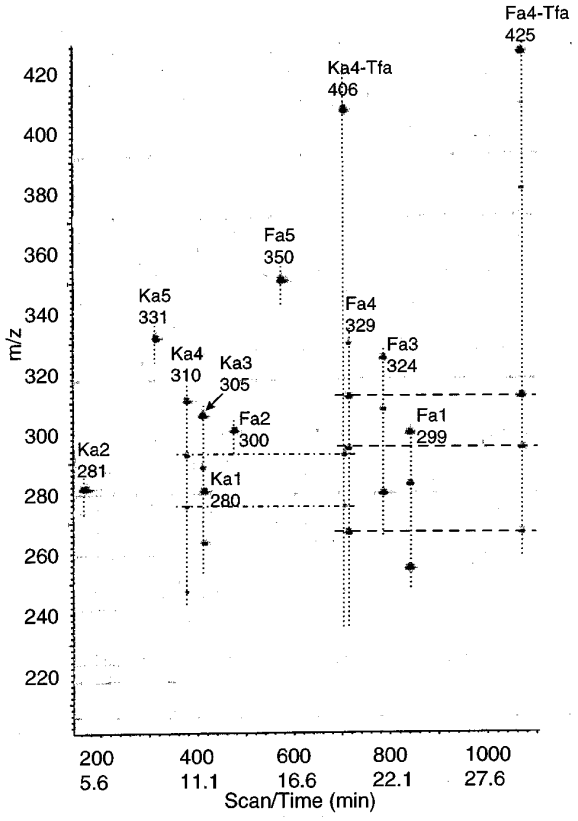

Figure 3. 2D plot of the HPLC-MS (in Figure 2) of the crude 2×5 aryl ether library. All displayed *m/z* values correspond to the $[M + H]^+$-ions of the protonated aryl ethers. Spots on a vertical dotted line indicate fragment ions or coeluting compounds; spots on a dashed horizontal line indicate isobaric fragment ions of the same nominal mass. The relationship of by-products and their corresponding aryl ethers is illustrated in the case of *Fa4-Tfa* by the horizontal dashed lines, and in the case of *Ka4-Tfa* by the horizontal dotted dashed lines.

Figure 4a shows the product ion spectrum of compound *Ka2* ($[M + H]^+$, *m/z* 281; top) and *Fa2* ($[M + H]^+$, *m/z* 300; bottom). The structural similarity of both aryl ethers is evident from the occurrence of the same fragment ions. The cleavage of the amide bond between NH and CO led to a fragment with *m/z* 135.5, which is characteristic of a 2-pyridyloxyacetyl group. The 2-pyridyloxymethylene fragment ion (*m/z* 108.0) is formed by a cleavage between CH_2 and CO (Figure 4a).

Similar fragmentation patterns were obtained with compounds *Ka5* ($[M + H]^+$, *m/z* 331; top) and *Fa5* ($[M + H]^+$, *m/z* 350; bottom) (Figure 4b). The cleavage of the amide bond between NH and CO resulted in

Table 2. Calculated monoisotopic and found masses of the $[M + H]^+$ ions in the split/combine and the premix library

No.	Code	Calculated mass (monoisotopic)	Found mass		No.	Code	Calculated mass (monoisotopic)	Found mass	
			Split/combine library	Premix library				Split/combine library	Premix library
1	Ka1	280.2	280.0	280.0	24	Rb2	365.2	365.1	365.0
2	Ka2	281.2	280.9	281.0	25	Kb4	366.2	366.1	366.0
3	Fa1	299.1	298.9	299.0[a]	26	Fc1	375.2	374.9	374.8[a]
4	Fa2	300.1	300.0	299.9	27	Fc2	376.2	376.0	–
5	Ka3	305.2	305.0	304.9	28	Fb3	380.2	380.0	–
6	Ra1	308.2	308.0	307.9	29	Kc3	381.2	381.1	381.0
7	Ra2	309.2	309.0	308.9	30	Rc1	384.2	384.1	384.0
8	Ka4	310.2	309.9	309.9	31	Rc2	385.2	385.0	385.0
9	Fa3	324.1	324.0	323.8	32	Fb4	385.2	385.0	385.0
10	Fa4	329.1	329.0	329.7	33	Kc4	386.2	386.1	386.0
11	Ka5	331.2	331.0	331.0	34	Kb5	387.2	387.0	387.0
12	Ra3	333.2	332.9	332.9	35	Rb3	389.2	389.1	389.0
13	Kb1	336.2	336.1	336.0	36	Rb4	394.2	–	–
14	Kb2	337.2	337.1	337.0	37	Fc3	400.2	400.0[b]	–
15	Ra4	338.2	338.1	338.0	38	Fc4	405.2	405.2[a]	405.2[a]
16	Fa5	350.1	349.9	349.9	39	Fb5	406.2	406.0	406.0
17	Fb1	355.2	355.1	355.0[a]	40	Kc5	407.2	407.1	407.0
18	Kc1	356.2	356.1	356.5	41	Rc3	409.2	409.0	409.0
19	Fb2	356.2	356.1	356.5	42	Rc4	414.2	413.6[c]	413.8[a]
20	Kc2	357.2	357.1	357.0	43	Rb5	415.2	415.0	415.1
21	Ra5	359.2	359.0	359.0	44	Fc5	426.2	426.0	426.0
22	Kb3	361.2	361.1	361.1	45	Rc5	435.2	435.0	435.0
23	Rb1	364.2	364.0	364.0					

All masses were determined in direct ESI-MS spectra, except where noted otherwise.
[a] Determined by NL MS/MS (constant neutral loss).
[b] Determined by LC-MS.

8-quinolinyloxyacetyl (*m/z* 185.5). Cleavage between CH_2 and CO produced a further fragment ion at *m/z* 157.5 (Figure 4b).

Corresponding fragmentation patterns as described in Figures 4a and b were not found with compounds *Ka4* and *Fa4* (Figure 4c). A loss of carboxamide $C(O)NH_2$ and H_2O from *Ka4* led to the fragment ion at *m/z* 246.5. In addition, cleavage of Lys-amide between CO and NH formed the fragment ion at *m/z* 163.5. Finally, the fragment ion at *m/z* 107 is formed by cleavage between CH_2 and CO of the phenoxy acetyl derivative and loss of the hydroxyl group.

The loss of C-terminal NH_3 and H_2O from the hydroxymethyl group in compound *Fa4* ($[M + H]^+$, *m/z* 329) resulted in a stable fragment ion at *m/z* 265.5 (Figure 4c, bottom). Cleavage between C^{α} and NH of the amino acid produced a Phe fragment ion at *m/z* 132.0. In this case, the substitution of Lys for Phe in the same aryl ether led to different fragmentation patterns.

The elucidation of by-products was also achieved by using product ion scans (Figure 4d). The occurrence of $[M + H]^+$-ions *Ka4+96* ($[M + H]^+$, *m/z* 406) and *Fa4+96* ($[M + H]^+$, *m/z* 425) indicated trifluoroacetylation of these compounds. The CID spectra of the trifluoroacetylated by-products (Figure 4d) contained similar fragment ions as described for the unmodified aryl ethers (Figure 4c). The fragmentation patterns verified that the additional mass of 96 u was located at the hydroxymethyl group. Thus, *Ka4* and *Fa4* must have been partially trifluoroacetylated at the free hydroxymethyl group during cleavage of the resin by TFA. *O*-Tfa esters are conveniently cleaved by treatment with aqueous ammonia followed by lyophilization. The formation of these by-products may be avoided by using a cleavage mixture containing aqueous TFA.

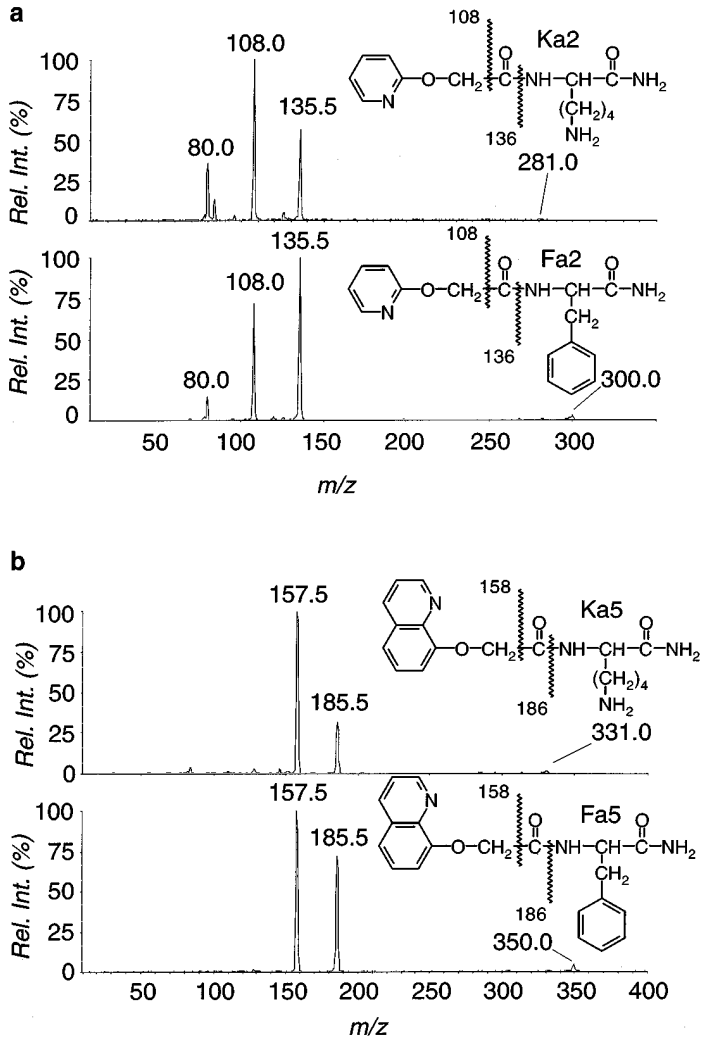

Figure 4. On-line tandem MS of the crude 2 × 5 aryl ether library. Product ion scans were recorded while the mixture eluted from the HPLC column (HPLC-MS/MS). The spectra show product ions of: (a) *Ka2* ($[M + H]^+$, *m/z* 281.0, top) and *Fa2* ($[M + H]^+$, *m/z* 300.0, bottom); (b) *Ka5* ($[M + H]^+$, *m/z* 331.0, top) and *Fa5* ($[M + H]^+$, *m/z* 350.0, bottom); (c) *Ka4* ($[M + H]^+$, *m/z* 310.0, top) and *Fa4* ($[M + H]^+$, *m/z* 329.0, bottom); (d) *O*-trifluoroacetylated by-products *Ka4-Tfa* ($[M + H]^+$, *m/z* 406.0, top) and *Fa4-Tfa* ($[M + H]^+$, *m/z* 425.0, bottom).

Mixtures of 45 aryl ethers

We investigated two molecule collections, both containing the same 45 members but synthesized either by the 'split/combine' or the 'premix' method (see above). In the case of an optimal synthesis, all aryl ethers should be present in equimolar amounts in both mixtures. Theoretically, both libraries should show identical peak and intensity patterns in the ESI-mass spectra of the protonated molecule ions. Therefore, differences in the mass spectra of the libraries are due only to synthetic effects. Furthermore, it has to be considered that the yield of ionization is dependent on the structure of the molecules. Some of the aryl ethers have identical nominal masses (so-called isobaric compounds, e.g. *Rc2* and *Fb4*, see Table 2), which should result in a doubled peak height (or more precisely peak area) in the ESI-MS spectrum compared to that of a compound present only once. Positively charged ions are easily obtained if the molecule contains basic functional groups, whereas the ease of ionization by protonation of hydrophobic molecules differs. In addition, the latter have a higher tendency to fragment, so that the peak height (area) of the resulting $[M + H]^+$-ion is much smaller. Especially in the case of low-molecular-mass

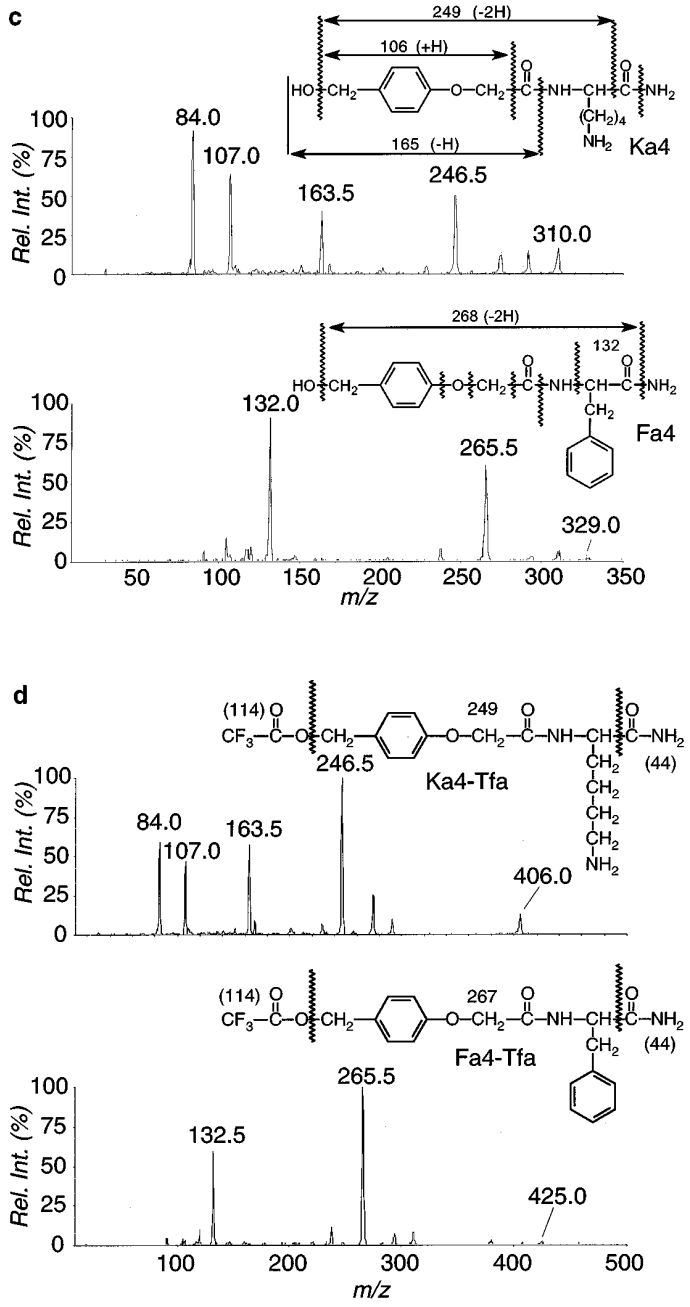

Figure 4. (Continued).

molecules these effects are much more pronounced than is observed for peptides. Therefore, due to structural differences, even if one of these mixtures contains an equimolar concentration of the aryl ethers, the peak heights (area) of the components are not necessarily the same in the ESI-MS spectrum of this library.

Direct ESI-MS

The ESI-MS spectra of the two collections showed a different mass and intensity distribution (Figure 5). This is the result of different concentrations of the aryl ethers (see above), caused by different nucleophilic displacement kinetics of the phenolates. If the library is

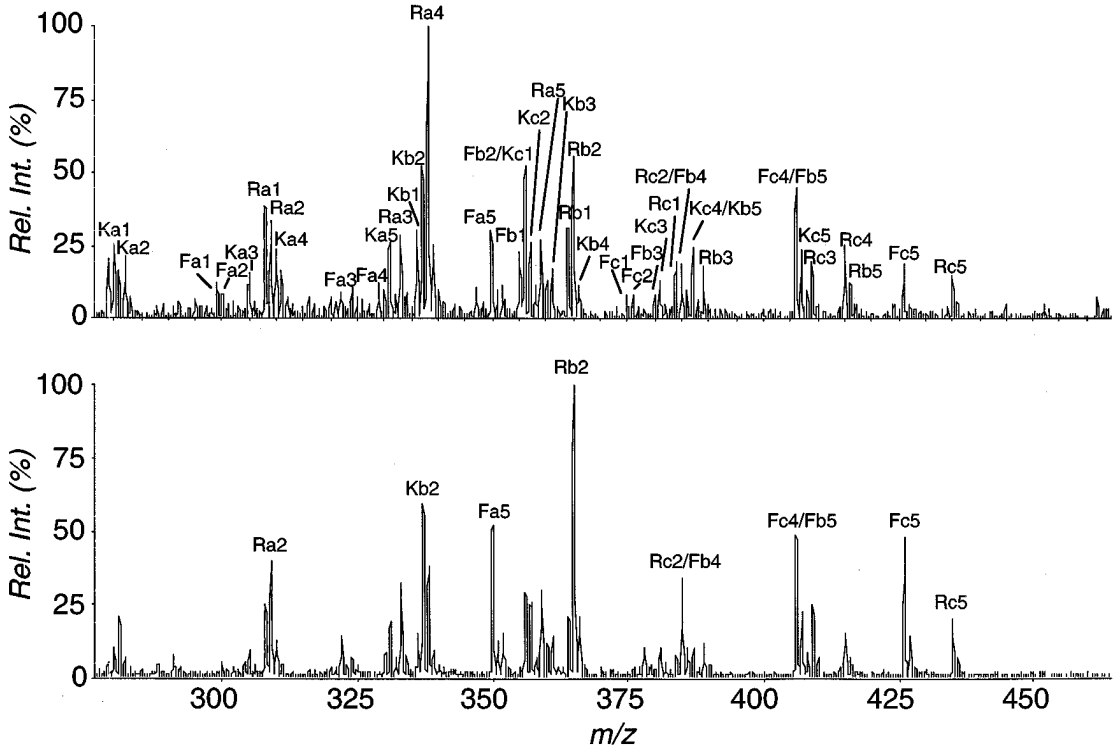

Figure 5. ESI-MS spectra of the crude 45 aryl ether libraries. Detected masses correspond to $[M + H]^+$-ions. Top: spectrum of the library synthesized by the split/combine method; bottom: spectrum of the library synthesized by the premix approach.

synthesized by the 'premix method', the different reaction rates may play a greater role than in the case of the 'split/combine' method. The individual intensities in each ESI-MS spectrum may differ mainly due to different yields of ionization. In fact, our analyses showed in the direct ESI-MS spectrum of the 'premix' library only the ions of 36 aryl ethers, nine aryl ethers (Table 2) were not found at all, whereas in the 'split/combine' library only four aryl ethers (Table 2) could not be detected (Figure 5).

Tandem MS

The complexity of ESI-mass spectra of mixtures can be reduced by tandem MS experiments. Figure 6a shows a precursor ion scan of the split/combine library. The *m/z* value of the second analyzer of the MS was set to the value of the product ion 2-pyridyloxymethylene (*m/z* 108.0). Only precursor ions which cleave this fragment ion are detected. Theoretically, 3×3 compounds contain the pyridyloxymethylene residue, but only the *Xa2* aryl ethers (X = *L*, *K*, *R*) are expected to eliminate this fragment ion caused by the cleavage of the labile bond between CH_2 and CO. For this reason the compounds *Xa2* are extracted out of the aryl ether mixture. Interestingly, one additional precursor ion (*Kb2*) eliminates this fragment ion by cleavage between two CH_2 groups of the hexanoic acid residue. In addition, the signal intensity of the $[M + H]^+$-ions of compounds *Ka2* and *Ra2* is much higher than that of *Fa2*, due to the basic amino acids Lys and Arg present in *Ka2* and *Ra2*. On the other hand, if the precursor ion shows a weak tendency to fragment, as in the case of *Kb2*, the signal intensity is low.

Another possibility to reduce the complexity of an ESI-MS spectrum is a constant neutral loss scan. We investigated both libraries by scanning the loss of Lys, Arg and Phe. For instance, in the case of Lys the mass difference between both analyzers was set to *m/z* 144 (Figure 6b). Thus, it should be possible to specifically extract ions containing Lys (15 ions). We observed that only 11 aryl ethers containing Lys were detected in this mode (Figure 6b), although all $[M + H]^+$-ions were found in the direct ESI-MS spectrum. It is conspicuous that *Ka3/Ka4* and *Kb3/Kb4* were missing, whereas all compounds of the *Kc*-series were detected. This result coincides with the fragmentation pattern we have found

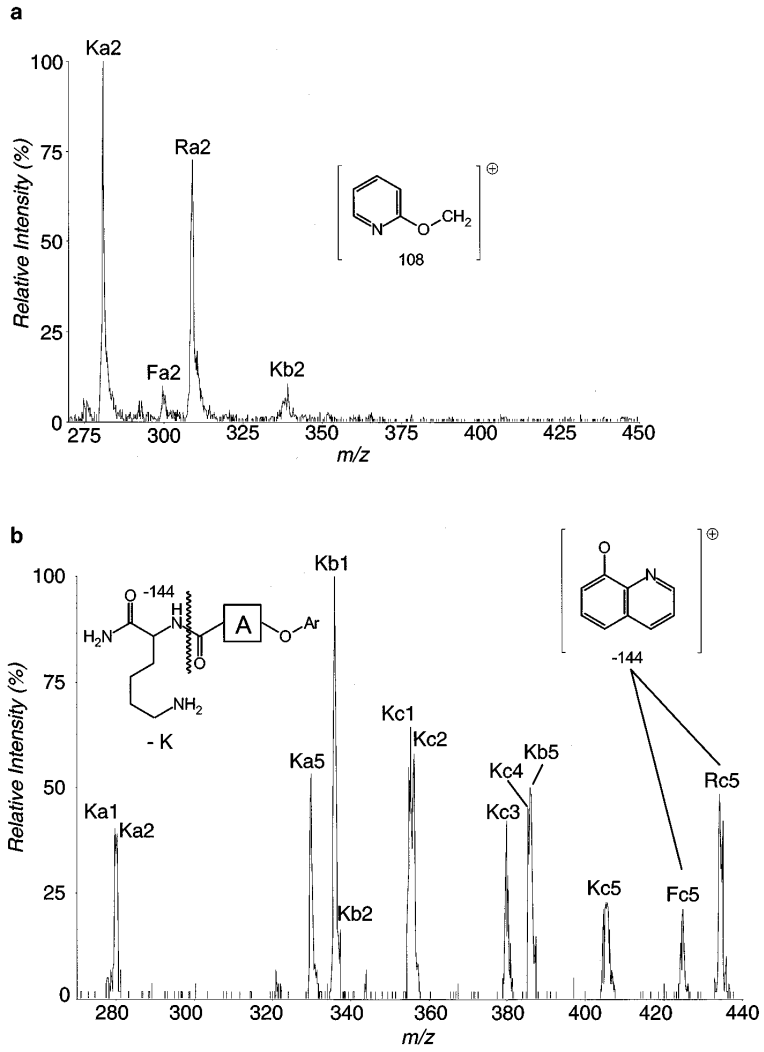

Figure 6. Tandem MS of the crude 45 aryl ether mixture synthesized by the split/combine method. (a) Precursor ion scan of *m/z* 108 (2-pyridyloxymethylene cation). The selected mass corresponds to the $[M + H]^+$-ions of the *Xa2*-series (*X* = *F, K, R*) and *Kb2*. (b) Constant neutral loss scan of *m/z* 144 (loss of Lys or 8-quinolinyloxy, respectively). The selected mass corresponds to the $[M + H]^+$-ions.

in the 2 × 5 library (Figure 4c). In compounds *Ka3/Ka4* and *Kb3/Kb4*, the bond between the C-terminal NH_2 and CO is cleaved first, so a loss of *m/z* 144 cannot be detected. In the case of the *Kc*-series, the loss of a Lys fragment is preferred, because there is a conjugation of the resulting positive charge of the cation over the aromatic system of the benzoic acid residue.

Moreover, two aryl ethers were detected which do not contain a Lys residue. The cleavage between CH_2 and O of the benzyl residue and 8-quinolinyloxy in the *Xc5*-series also results in a fragment ion at *m/z* 144 (Figure 6b). For this reason the aryl ethers *F/Rc5* are also extracted out of the library by this tandem experiment.

Some aryl ethers, which could not be detected in direct measurements, were discovered by tandem MS experiments (Table 2). For example, most of the missing aryl ethers in the direct ESI-mass spectrum of the premix library contain Phe and were determined by a constant neutral loss scan (loss of Phe, *m/z* 163) (Table 2).

HPLC-MS

We investigated the aryl ether mixture obtained by the split/combine method by RP-HPLC-MS. Figure 7

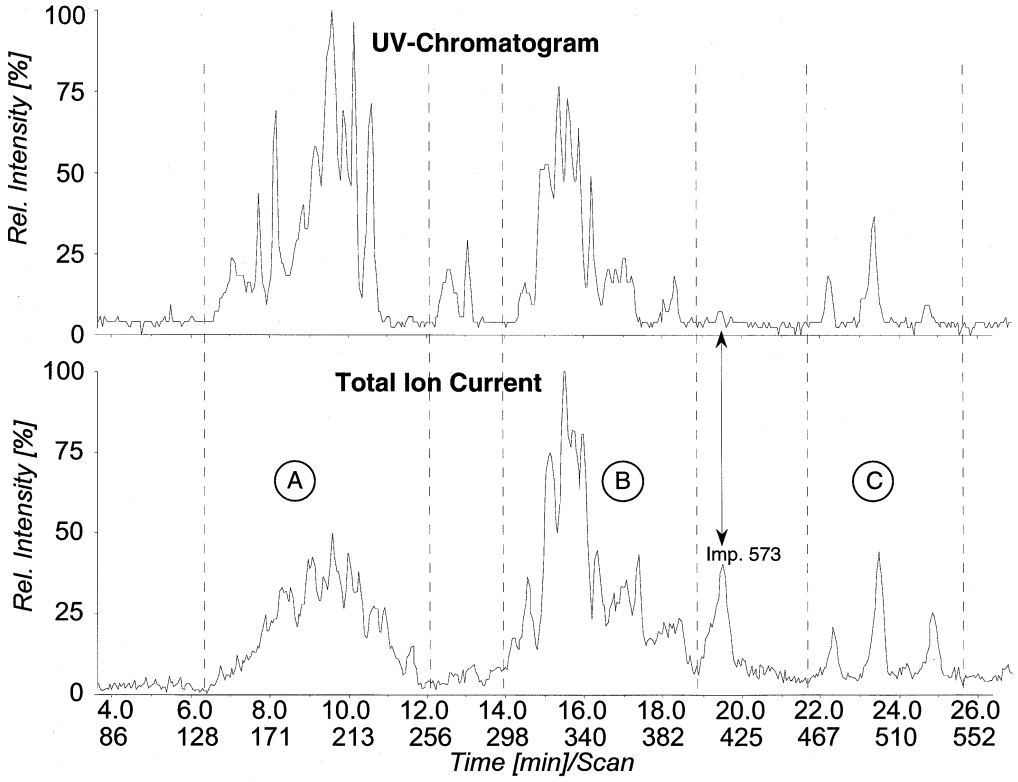

Figure 7. UV trace (detection at 214 nm) and a TIC of the crude 45 aryl ether mixture synthesized by the split/combine method. The chromatograms can be divided into three areas A, B and C. An impurity (*m/z* 573), which shows no UV absorbance, is indicated with an arrow.

shows the UV-trace (detection at 214 nm) and the TIC, which can be divided into three groups A, B and C. A good correlation of the TIC with the UV trace was obtained. In the first group A, no compounds containing a Phe residue are found due to their greater lipophilicity. In contrast, the last eluting group C embodied the most nonpolar molecules *Fb3*, *Fc3/Fb*1 and *Fc1* (see also Figure 8). An unknown impurity (*m/z* 573) showed an intense signal in the TIC, whereas almost no UV signal was detected. An unambiguous assignment of the masses found in the 2D plot to their corresponding aryl ethers was impossible due to the strong fragmentation tendency. For this reason, the discovery of by-products is more difficult than in the case of the 2 × 5 library. Even a semiquantitative evaluation of the library's composition was impossible with this experiment.

Figure 8 shows the 2D contour plot of section C (Figure 7). Due to the group's 'isolated' position in the TIC, it was possible to identify the library members *Fb3*, *Fc3/Fb1* and *Fc1*. We also observed a coelution of compound *Fc3* ($[M + H]^+$, *m/z* 400.0) with *Fb1* ($[M + H]^+$, *m/z* 355.0). All nonpolar compounds in group C showed a similar fragmentation pattern which involved the loss of NH_3 ($[M + H - 17^+]$) caused by cleavage between C-terminal NH_2 and CO and the loss of carboxamide $C(O)NH_2$ ($[M + H - 45^+]$) (Figure 8). Such a pronounced tendency to fragment is often observed in ESI-MS measurements of more hydrophobic small molecules [13].

Conclusions

Impurities or missing components in small molecule libraries used in biological assays may endanger the success of the whole library approach. Whereas most activities in combinatorial chemistry are focused on the synthesis, so far only limited attempts have been made to confirm the identity of the mixtures. This, however, is of utmost importance for the optimization of a synthetic strategy. Due to different ionization yields as well as the relatively strong fragmentation of small molecules, the analysis of ESI-mass spectra of small

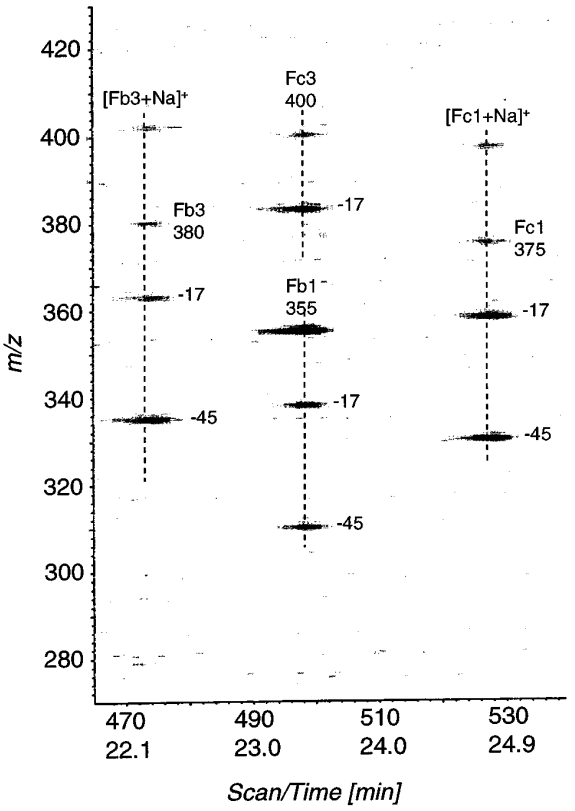

Figure 8. 2D contour plot of area C shown in Figure 7. All displayed *m/z* values correspond to the $[M + H]^+$-ions of the protonated aryl ethers. Vertical dashed lines indicate fragment ions or coeluting compounds. Displayed negative masses are mass differences between the $[M + H]^+$-ions and the detected fragment ions.

molecule libraries is not as straightforward as the mass spectrometric analysis of e.g. peptide libraries. The ESI-mass spectra of the aryl ether mixtures presented here give basically qualitative information. In the case of the 2×5 library, RP-HPLC-ESI-MS also allowed us to obtain at least semiquantitative information on the composition. By using tandem MS with product ion scans, the identification of library members, as well as by-products derived thereof, was confirmed. It could be shown that the small 2×5 library contained two by-products in considerable amount. Using other analytical techniques, these results cannot be obtained with comparable reliability, accuracy and rapidity. In the 45 aryl ether libraries some components, most of them containing a Phe residue, were not detected in the direct ESI-mass spectrum. However, most of these aryl ethers were detected when using tandem MS. Molecules containing Lys and Arg residues are readily ionized due to their basic side chain, whereas the ionization yield of aryl ethers with a Phe residue is lower. These effects play a greater role in small molecule libraries than in the case of peptide libraries. Only qualitative information on the composition of the 45 aryl ether libraries was obtained. In particular, tandem MS is a very powerful tool for reducing the complexity of ESI-mass spectra achieved from small molecule libraries. It can be used for the analysis of by-products as well as for the 'extraction' of sublibraries with common structural features. In summary, the obtained information allows a very rapid optimization of the synthesis including solid-phase reactions, cleavage and purification steps. Our latest results show that it is possible to obtain qualitative information also on larger small molecule libraries containing up to 400 components (fingerprint spectra [23,24]). If a defined residue in the library is substituted for another one, the patterns of the mass spectra, the so-called mass spectrometric fingerprints of the library, containing peaks of the protonated molecule ions and fragments, are shifted according to the mass difference of the residues.

Acknowledgements

This work was supported by the Bundesministerium für Bildung, Forschung und Technologie BMBF project 03 D 0037A and the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich SFB 323). We wish to thank our students Stephan Meier and Alf Herzig for technical assistence in library synthesis.

References

1. Gordon, E.M., Barrett, R.W., Dower, W.J., Fodor, S.P.A. and Gallop, M.A., *Applications of combinatorial technologies to drug discovery. 2. Combinatorial organic synthesis, library screening strategies, and future directions*, J. Med. Chem., 37 (1994) 1385–1401.
2. Jung, G. (Ed.) *Combinatorial Peptide and Non-peptide Libraries*, Verlag Chemie, Weinheim, Germany, 1996.
3. Desai, M.C., Zuckermann, R.N. and Moos, W.H., *Recent advances in the generation of chemical diversity libraries*, Drug Dev. Res., 33 (1994) 174–188.
4. Gordon, E.M., Gallop, M.A. and Patel, D.V., *Strategy and tactics in combinatorial organic synthesis. Applications to drug discovery*, Acc. Chem. Res., 29 (1996) 144–154.
5. Früchtel, J.S. and Jung, G., *Organic chemistry on solid support*, Angew. Chem., 108 (1996) 19–46; Angew. Chem., Int. Ed. Engl., 35 (1996) 17–42.
6. Thompson, L.A. and Ellman, J.A., *Synthesis and applications of small molecule libraries*, Chem. Rev., 96 (1996) 555–600.
7. Hermkens, P.H.H., Ottenheijm, H.C.J. and Rees, D., *Solid-phase organic reactions: A review of the recent literature*,

Tetrahedron, 52 (1996) 4527–4554.
8. Hobbs-DeWitt, S., Schroeder, M.C., Stankovic, C.J., Strode, J.E. and Czarnik, A.W., *DIVERSOMER technology: Solid phase synthesis, automation, and integration for the generation of chemical diversity*, Drug Dev. Res., 33 (1994) 116–124.
9. Dankwardt, S.M., Phan, T.M. and Krstenansky, J.L., *Combinatorial synthesis of small-molecule libraries using 3-amino-5-hydroxybenzoic acid*, Mol. Div., 1 (1995) 113–120.
10. Scott, B.O., Siegmund, A.C., Marlowe, C.K., Pei, Y. and Spear, K.L., *Solid phase organic synthesis (SPOS): A novel route to diketopiperazines and diketomorpholines*, Mol. Div., 1 (1995) 125–134.
11. Dunayevskiy, Y., Vouros, P., Carell, T., Wintner, E.A. and Rebek Jr., J., *Characterization of the complexity of small-molecule libraries by electrospray ionization mass spectrometry*, Anal. Chem., 67 (1995) 2906–2915.
12. Dunayevskiy, Y.M., Vouros, P., Wintner, E.A., Shipps, G.W., Carell, T. and Rebek Jr., J., *Application of capillary electrophoresis-electrospray ionization mass spectrometry in the determination of molecular diversity*, Proc. Natl. Acad. Sci. USA, 93 (1996) 6152–6157.
13. Metzger, J.W., Kempter, C., Wiesmüller, K.-H. and Jung, G., *Electrospray mass spectrometry and tandem mass spectrometry of synthetic multicomponent peptide mixtures: Determination of composition and purity*, Anal. Biochem., 219 (1994) 261–277.
14. Metzger, J.W., Wiesmüller, K.-H., Gnau, V., Brünjes, J. and Jung, G., *Ion-spray mass spectrometry and high performance liquid chromatography – mass spectrometry of synthetic peptide libraries*, Angew. Chem., 105 (1993) 901–903; Angew. Chem., Int. Ed. Engl., 32 (1993) 894–896.
15. Metzger, J.W. and Eckeskorn, C., *Microcharacterisation of proteins*, In Kellner, R., Lottspeich, F. and Meyer, H.E. (Eds.), VCH, Weinheim, Germany, 1994, pp. 167–188.
16. Rano, T.A. and Chapman, K.T., *Solid phase synthesis of aryl ethers via the Mitsunobu reaction*, Tetrahedron Lett., 36 (1995) 3789–3792.
17. Krchňák, V., Flegelova, Z., Weichsel, A.S. and Lebl, M., *Polymer-supported Mitsunobu ether formation and its use in combinatorial chemistry*, Tetrahedron Lett., 36 (1995) 6193–6196.
18. Krchňàk, V., Weichsel, A.S., Cabel, D., Flegelova, Z. and Lebl, M., *Structurally homogeneous synthetic combinatorial libraries*, Mol. Div., 1 (1995) 149–164.
19. Krchňàk, V., Weichsel, A.S., Issakova, O., Lam, K.S. and Lebl, M., *Bifunctional scaffolds as templates for synthetic combinatorial libraries*, Mol. Div., 1 (1995) 177–182.
20. Wiesmüller, K.-H., Udaka, K., Kienle, S., Walden, P. and Jung, G., *Functional determinants of MHC class I-peptide and T-cell receptor-MHC-peptide interactions defined with peptide libraries*, In Maia, H.L.S. (Ed.) Peptides 1994 (Proceedings of the 23rd European Peptide Symposium), ESCOM, Leiden, The Netherlands, 1995, pp. 111–112.
21. Kienle, S., Udaka, K., Walden, P., Feiertag, S., Kempter, C., Metzger, J.W., Jung, G. and Wiesmüller, K.-H., *Chemical and biological quality of CTL epitope modified in a stepwise fashion from defined positions to mixture position*, In Maia, H.L.S. (Ed.) Peptides 1994 (Proceedings of the 23rd European Peptide Symposium), ESCOM, Leiden, The Netherlands, 1995, pp. 863–864.
22. Furka, A., Sebestyen, F., Asgedom, M. and Dibo, G., *A general method for the rapid synthesis of multicomponent peptide mixtures*, Int. J. Pept. Protein Res., 37 (1991) 487–493.
23. Haap, W.J. and Jung, G., *Solid-phase synthesis of complex benzylcyanide collections*, manuscript submitted.
24. Früchtel, J.S., Haap, W.J., Trautwein, A.W., Walk, T. and Jung, G., *Solid-phase synthesis of pyrrolidines*, manuscript submitted.