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Application of capillary electrophoresis–electrospray ionization mass spectrometry in the determination of molecular diversity

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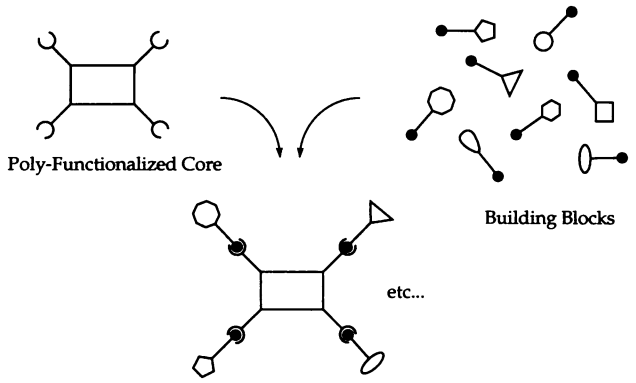
ABSTRACT **By means of capillary electrophoresis coupled online to electrospray ionization MS, a library of theoretically 171 disubstituted xanthene derivatives was analyzed. The method allowed the purity and makeup of the library to be determined: 160 of the expected compounds were found to be present, and 12 side-products were also detected in the mixture. Due to the ability of capillary electrophoresis to separate analytes on the basis of charge, most of the xanthene derivatives could be resolved by simple capillary electrophoresis–MS procedures even though 124 of the 171 theoretical compounds were isobaric with at least one other molecule in the mixture. Any remaining unresolved peaks were resolved by MS/MS experiments. The method shows promise for the analysis of small combinatorial libraries with fewer than 1000 components.**

Recently, the field of combinatorial chemistry has attracted much interest in the scientific community. The ability to synthesize large pools of novel compounds and screen them simultaneously for biological activity is likely to be a powerful asset in the search for lead drug candidates (1–4). Current trends in combinatorial chemistry have tended toward the creation of nonpeptide libraries, because peptides have limitations as potential therapeutic agents. One method of obtaining libraries of small organic molecules is to combine a core molecule with a varied set of building blocks; in this way, different functional groups are added to a scaffold structure with multiple reactive sites (Scheme I).



SCHEME I

The shape of the scaffold and the functionality of the building blocks can each be optimized to meet specific structural objectives. Carell *et al.* (5, 6) have reported the use of cubane and xanthene core molecules to generate libraries with a diversity of 10^4-10^5 different molecules.

The above methodology makes use of solution-phase synthesis, obviating the need for solid-support chemistry. However, because common synthetic problems, such as incomplete coupling of building blocks, formation of by-products, etc., would be even more pronounced in a solution-phase procedure than on a solid support, an analysis of libraries is desired to determine the complexity and composition of the mixtures produced. Knowledge of the quality of the libraries generated is essential to determine the likelihood of false negatives or false positives when screening a combinatorial library for useful compounds. In the case of false negatives, a potential ligand could be missed in the screening procedure either because it was not present or because it was present in a very low amount. False positives, on the other hand, could be caused by the activity of side-products.

Electrospray ionization MS (ESI-MS) is a very powerful tool for the analysis of biomolecules, providing both the molecular weight and structural characterization of analytes. When coupled to a separation technique, the capability of MS is greatly enhanced, and the hyphenated system provides an excellent method for the analysis of combinatorial libraries. The combinatorial libraries that we wished to analyze consist of hydrophilic charged molecules bearing multiple functionalities such as carboxylate and ammonium groups. Their polarity ruled out a normal phase separation technique, and tests showed that use of reverse-phase liquid chromatography provided poor separation of species containing one or more carboxylate groups. What was needed was a separation based on charge in aqueous solution.

In this paper we describe the use of capillary electrophoresis (CE) coupled online with ESI-MS (7, 8) to probe the synthesized combinatorial libraries for their diversity. CE generates rapid, high-resolution separations based on differences in the electrophoretic mobilities of charged species in an electric field in small-diameter fused-silica capillaries. Application of CE before mass spectrometric detection, therefore, provides fast and efficient separation of library components for their subsequent characterization by ESI-MS.

MATERIALS AND METHODS

Synthesis of Combinatorial Libraries. Except for the synthesis of the xanthene core **3**, detailed below, the method of preparation of the combinatorial libraries used herein has already been described (5, 6, 9). In brief, the given acid chloride core is condensed with an equimolar mixture of amines in dichloromethane and triethylamine, and the resulting dichlo-

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Abbreviations: ESI-MS, electrospray ionization MS; CE, capillary electrophoresis; EOF, electroosmotic flow.

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romethane-soluble product mixture is extracted first with a citric acid solution and then with water. The protected library thus formed is deprotected by stirring 8 h in reagent K: trifluoroacetic acid/water/phenol/thioanisole/ethanedithiol (82.5:5:5:5:2.5, vol/vol) (10). The deprotected mixture is concentrated *in vacuo*, precipitated by addition of cold ether/*n*-hexanes (1:1), and filtered, yielding the library as a powder.

9,9-Dimethylxanthene-4,5-dicarboxylic acid. 9,9-Dimethylxanthene (2.00 g, 9.5 mmol; obtained from Aldrich), dry heptane (100 ml), and *N,N,N′,N′*-tetramethylethylenediamine (TMEDA; 3.5 ml, 24 mmol) were combined and degassed with argon for 15 min before *n*BuLi (1.6 M in 15 ml of hexane, 24 mmol) was added (11). The solution was warmed to reflux under argon for 15 min, then allowed to cool to room temperature. The reddish solution was cannulated onto a large excess of dry ice and allowed to stand for 1 h, then ethyl acetate and 2 M HCl were added until most of the solids dissolved and the pH reached 1. The layers were separated, the aqueous layer was extracted twice with ethyl acetate/tetrahydrofuran (20:1), and the combined organic layer was rinsed once with brine and dried over sodium sulfate. Upon concentration, a white solid precipitated, and the flask was then cooled to 0°C and the product was filtered off. A slightly yellow powder (mp 248–249°C), pure by NMR and weighing 1.39 g (49%), was isolated. Additional material (≈0.2 g) could be recovered from the yellow-orange filtrate but contained at an impurity of ≈15% of an impurity. ^{1}H NMR (250 MHz, dimethyl sulfoxide, ppm): δ = 12.9 (brs, 2 H), 7.80 (dd, J = 7.8 and 1.7 Hz, 2 H), 7.69 (dd, J = 7.7 and 1.5 Hz, 2 H), 7.23 (t, J = 7.8 Hz, 2 H), 1.61 (s, 6 H). ^{13}C NMR (62.9 MHz, dimethyl sulfoxide, ppm): δ = 166.19, 147.72, 130.72, 130.40, 129.45, 123.57, 119.93, 33.85, 31.94. High-resolution MS (electron impact) calculated for $C_{17}H_{14}O_5$, 298.0841; found, 298.0839.

9,9-Dimethylxanthene-4,5-dicarboxylic acid chloride. To the suspended diacid (0.75 g, 0.25 mmol) at 0°C in ethanol-free chloroform (100 ml) was added oxalyl chloride (4.0 ml, 46 mmol) and dimethylformamide (5 ml). Within 15 min a clear solution was evident, and the contents were warmed to reflux under a drying tube for 1 h. The solution was evaporated to dryness, 5 ml of dry toluene was added, and it was evaporated again. The pale yellow solid (mp 144–146°C) was pure by NMR. ^{1}H NMR (250 MHz, C^2HCl_3, ppm): δ = 7.92 (dd, J = 7.7 and 1.5 Hz, 2 H), 7.68 (dd, J = 7.9 and 1.5 Hz, 2 H), 7.25 (t, J = 7.8, 2 H), 1.66 (s, 6 H). ^{13}C NMR (62.9 MHz, C^2HCl_3, ppm): δ = 163.55, 163.34, 131.57, 131.22, 131.11, 123.65, 34.41, 31.99. High-resolution MS (electron impact) calculated for $C_{17}H_{12}Cl_2O_3$, 334.0163; found, 334.0160.

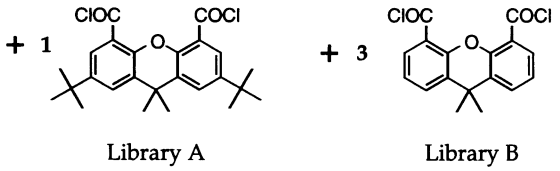
CE–ESI-MS. Experiments were performed on a triple quadrupole mass spectrometer TSQ-700 (Finnigan-MAT, San Jose, CA) equipped with a Finnigan atmospheric pressure ionization source operated in the electrospray ionization mode. The first and third quadrupoles of the instrument served as independent analyzers, and the second region was utilized as a collision chamber for MS/MS experiments. Ion optic settings and sheath gas pressure were optimized on the day of the analysis. For positive ion detection, the electrospray voltage was maintained at +4.5 kV, and the temperature of the capillary was held at 150°C. The effective mass range was from m/z = 500 to m/z = 1000 in normal scan mode at a rate of 1.5 s per scan. Determination of the structures of the selected compounds was performed using collision-induced dissociation with 1 mtorr (1 torr = 133 Pa) of argon and collision energy setting of −50 V. Fragment ions were detected in the range from m/z = 50 to the anticipated parent ion mass.

The CE capillary tubing consisted of uncoated fused silica with the following dimensions: o.d., 375 mm; i.d., 50 mm; and length, 40 cm. The electrophoresis buffer was 20 mM Tris in water, adjusted to a pH of 7.9 with acetic acid. The liquid sheath was 1 mM Tris acetate (pH 7.9) in H_2O/MeOH (25:75, vol/vol) with a flow rate of 1.5–2.5 ml/min. Liquid sheath flow rate was optimized to maintain a stable spray and to achieve the maximum analyte response and efficient CE separation conditions; nitrogen gas was used to stabilize the spray, and its pressure varied from 0.1 to 1 psi (1 psi = 6.89 kPa). The injection end of the capillary was held at a positive potential of +24.5 kV with a resultant current of 9 μA. The potential drop across the capillary was 20 kV (500 V/cm), because the anode end was at the same voltage as the electrospray needle—i.e., +4.5 kV. The library samples were injected hydrodynamically at a height of 15 cm for 10 s, which corresponded to injection volumes of 5 nl. The CE interface was configured in-house, and its description can be found elsewhere (12).

RESULTS

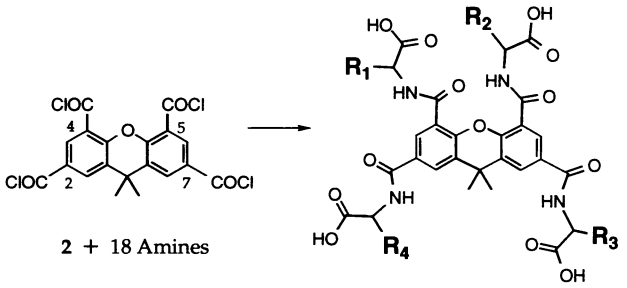
The library to be tested was constructed with core molecule **1** and a set of 18 amine building blocks (Scheme **II**, Library A).

	amino acid	protected reagent used
1	(Gly)-OMe	Glycine-methyl ester
2	(Ala)	L-alanine-*tert*-butyl ester
3	(Ser)	O-*tert*-butyl-L-serine-*tert*-butyl ester
4	(Pro)	L-proline-*tert*-butyl ester
5	(Val)	L-valine-*tert*-butyl ester
6	(Leu)	L-leucine-*tert*-butyl ester
7	(Ile)	L-isoleucine-*tert*-butyl ester
8	(Asn)	L-asparagine-*tert*-butyl ester
9	(Asp)	L-aspartic acid-β-*tert*-butyl-α-*tert*-butyl ester
10	(Thr)-OMe	O-*tert*-butyl-L-threonine-methyl ester
11	(Glu)	L-glutamic acid-γ-*tert*-butyl-α-*tert*-butyl ester
12	(His)	N^{im}-trityl-L-histidine
13	(Lys)-OMe	N^{ε}-Boc-L-lysine-methyl ester
14	(Met)-OMe	L-methionine-methyl ester
15	(Phe)	L-phenylalanine-*tert*-butyl ester
16	(Arg)	N^{g}-4-methoxy-2,3,6-trimethylbenzene -sulfonyl-L-arginine
17	(Trp)-OMe	L-tryptophane-methyl ester,
18	(Tyr)-OMe	O-*tert*-butyl-L-tyrosine-methyl ester,



SCHEME II

After formation of the library, the protecting groups shown in Scheme **II** were removed with the trifluoroacetic acid-based reagent K (see *Materials and Methods*) to leave the free amino acid functionalities. This library was synthesized to examine the quality of a larger library that had been used to screen for a trypsin inhibitor (9) (Scheme **III**).



SCHEME III

The large library used for screening had been constructed by condensing core molecule **2** with the same eighteen amines, and it was assumed that any hindrance to formation of

particular compounds in the library would result from interactions at the closely located 4 and 5 positions of **2**. Thus, the core molecule **1** was a logical choice for testing the synthetic viability of Scheme **III**. The presence of two highly hydrophobic *tert*-butyl groups at positions 2 and 7 allowed evaluation of the precipitation behavior of hydrophobic library components during the final ether/*n*-hexane treatment step. The results of these analyses were compared to the results obtained for a library of the same 18 amines constructed with core molecule **3** (Scheme **II**, library B), which did not contain *tert*-butyl groups at positions 2 and 7.

Previously we described the characterization of libraries containing up to 55 compounds using ESI-MS alone—i.e., without prior separation of the mixture (13). However, application of MS alone for more complex mixtures is limited because a significant number of ions may be present within a fairly narrow mass range. Additionally, competition of a large number of different ions for protons during electrospray ionization may cause a decrease in sensitivity and discrimination between analytes of different basicity. To analyze a library constructed with core molecule **1** and 18 amines—a library of theoretically 171 compounds, many of which are isobaric—it became necessary to add another separation dimension to our MS analysis. To that effect, we explored the use of CE coupled online to tandem MS, because CE offers high speed and high-resolution separation.

CE acts by differentiating between charged molecules, and the charge of the di-amino-acid-substituted xanthene derivatives was determined by the derivative of the carboxyl terminus and the side chain of the amino acid attached to each core molecule. Under the buffer conditions used, termini were either negatively charged carboxylates or neutral methyl esters, and the charges on the side chains could be positive, neutral, or negative (e.g., lysine, valine, and glutamic acid, respectively). Thus, the variety of building blocks used afforded a diversity of charges in the library components.

The use of an uncoated capillary was preferable, because this allowed all of the analytes to be detected in one run. The inner walls of the bare silica capillary are negatively charged under aqueous conditions due to acidic silanol groups, and this causes the formation of an excess of positive charges of the electrophoretic buffer layer in contact with the inner wall. When voltage is applied across the capillary, electroosmotic flow (EOF) moves the sample from the positive end (anode) to the negative end (cathode). EOF becomes significant at pH values >5 and drives the analytes toward the cathode, regardless of their charge. Thus, cations, neutrals, and anions can be electrophoresed in a single run because they all move in the same direction. The resultant mobility of the library compounds and therefore their migration times are determined by the difference between the mobility of the EOF and the electrophoretic mobility of the species. The electrophoretic conditions could be chosen to separate a complex mixture into several groups of analytes, each migrating according to their charge. Within each such group one can expect significantly fewer isobaric compounds compared with the (unresolved) total library mass spectrum.

Several small representative sublibraries were analyzed in order to investigate the electrophoretic behavior of xanthene

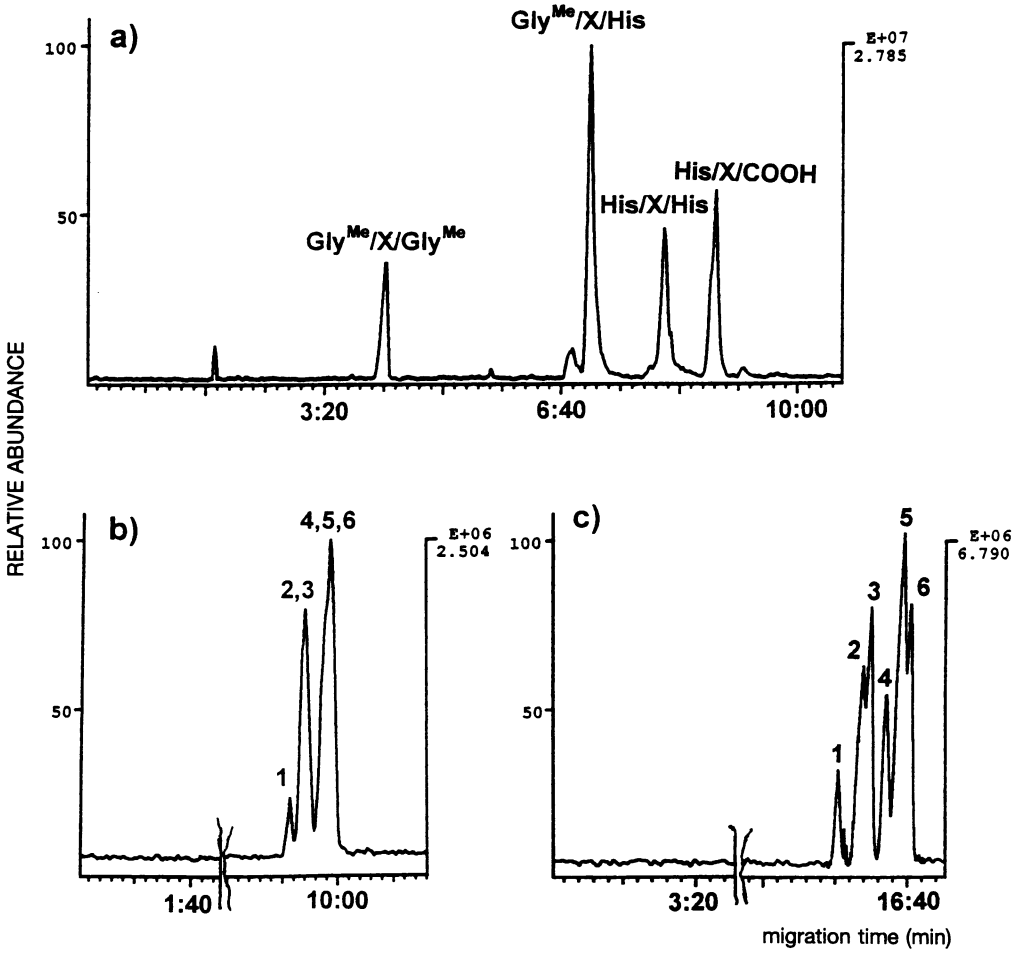


FIG. 1. CE–MS electropherogram of different mixtures of xanthene derivatives in Tris/acetate buffer at pH 7.9. (*a*) Mixture 1, Gly^{Me}/X/Gly^{Me}, Gly^{Me}/X/His, and His/X/His. (*b*) Mixture 2, Ile/X/Ile (**1**), Ile/X/Pro (**2**), Ile/X/Ala (**3**), Pro/X/Pro (**4**), Pro/X/Ala (**5**), and Ala/X/Ala (**6**). (*c*) Mixture 2, dissolved in 20 mM Tris/acetate buffer at pH 7.9 containing 40% (vol/vol) MeOH. E+07 indicates 10^7.

derivatives and optimize CE–MS conditions for their detection. Fig. 1 shows one such analysis for two simple mixtures. The first sublibrary consisted of three compounds (from core molecule **1**, abbreviated X, plus two amino acids: Gly^{Me} and His), and the second sublibrary consisted of six compounds (from core **1** plus three amino acids: Ala, Pro, and Ile).

In Fig. 1*a*, noncharged di(glycine methyl ester)-substituted xanthene (Gly^{Me}/X/Gly^{Me}) comigrated with the EOF, followed by Gly^{Me}/X/His, His/X/His, and His/X/COOH. (His/X/COOH was a significant impurity in which the xanthene was only monosubstituted.) In the electropherogram of Fig. 1*b*, disubstituted xanthenes were only partially separated, because they were all similar, with two hydrophobic side chains and two negatively charged carboxyl termini. In spite of the close migration times, all six analytes were clearly observed from their mass spectra, which contained only protonated molecules. The first peak in that group corresponded to Ile/X/Ile, the second to Ile/X/Pro and Ile/X/Ala, and the last to Pro/X/Pro, Pro/X/Ala, and Ala/X/Ala. The electrophoretic separation of charged species is based on their charge-to-mass ratio ($\mu \approx z/m^{2/3}$ where μ = electrophoretic mobility, z = charge of the analyte, and m = mass of the analyte) (14). Therefore, separation by CE can be achieved even for the similarly charged compounds (compare Ile/X/Ile and Ala/X/Ala in Fig. 1*b*).

There are several options available to improve the separation of the analytes by CE. Among those are buffer composition, pH, and ionic strength, all of which were investigated in order to optimize electrophoretic separation. The larger velocity of the EOF at high buffer pH caused fast elution of the analytes, lowering resolution even though the compounds were more negatively charged under these conditions. At pH 9, the six compounds, which were partially resolved in Fig. 1*b* (pH 7.9), eluted earlier (at about 6 min) as one broad unresolved peak. Decrease in buffer pH below 7.9 did not improve the separation of the compounds but only resulted in their slower migration and longer analysis time. The change of ionic strength of the buffer showed that 50 mM Tris buffer offered better separation with longer runs, and 10 mM Tris buffer gave faster but poorer separation. Additionally, a high concentration of Tris caused ion signal suppression in the electrospray ionization process. The electrophoretic conditions finally chosen for analysis of the xanthene libraries were 20 mM Tris buffer at pH 7.9. Resolution could be significantly improved by introduction of an organic modifier in the CE buffer (15). When 40% MeOH was added to the CE buffer, all six components of the mixture in Fig. 1*b* were separable (Fig. 1*c*). However, addition of methanol greatly increased the analysis time; for example, use of 50% MeOH in the CE buffer provided for complete resolution of the xanthene derivatives but with a total run time of 30 min. Therefore, an organic modifier can be used in a situation where poor separation hinders the full characterization of the synthesized large libraries.

Fig. 2*d* shows the full CE–MS electropherogram for library A (Scheme **II**), with three selected molecular ion traces a, b, and c displayed above. The first peak, comigrating with the EOF, consisted of noncharged derivatives that were substituted with amino acid methyl esters on both sites of the core molecule **1**. Most of the compounds with lysine methyl ester on one side and a neutral amino acid on the other also came out with this peak, because the positive charge of the lysine side chain balanced the negative charge on the carboxyl of the second building block. The next group of peaks (from 6 to 7.5 min) consisted of various compounds substituted with arginine and also compounds containing both an amino acid methyl ester and a neutral amino acid (with a free carboxyl terminus). The third group in the electropherogram (from 8 to 10 min) corresponded to those derivatives with two neutral amino acids plus derivatives substituted with one acidic amino acid (e.g.,

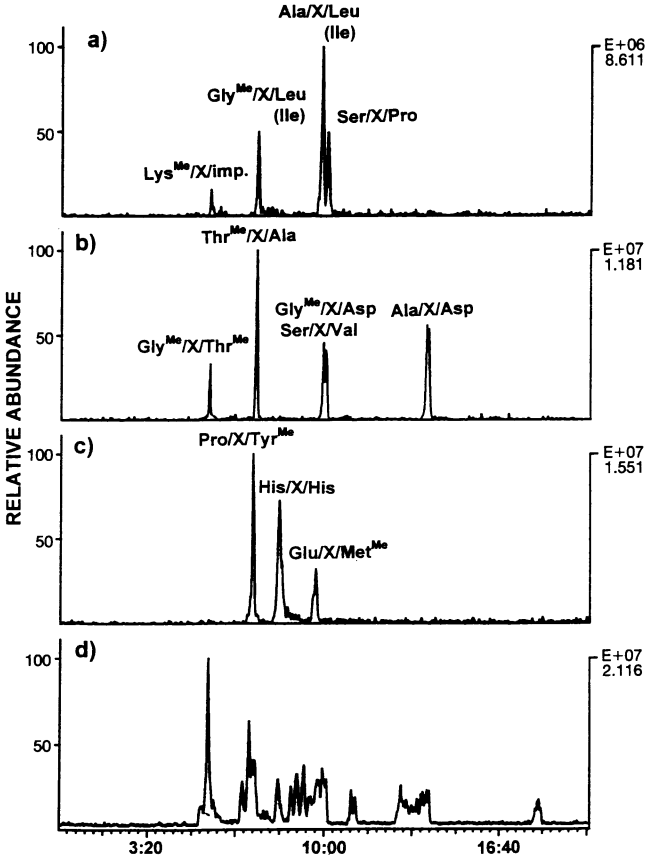


FIG. 2. CE–MS electropherogram of library A with extracted ion electropherograms. (*a*) m/z = 595.5. (*b*) m/z = 597.5. (*c*) m/z = 685.5. (*d*) Total ion current electropherogram.

Glu) and one neutral amino acid methyl ester. His/X/Asp and His/X/Glu migrated at 11 min, followed by another group of peaks containing compounds of one acidic and one neutral amino acid. The last peak consisted of three derivatives with negatively charged amino acids: Asp/X/Asp, Asp/X/Glu, and Glu/X/Glu. Thus, library components were distributed within a rather wide migration time frame, allowing identification of the analytes in each group by subsequent MS.

While most of the isobaric compounds in the mixture were separated by CE (e.g., the analytes in Fig. 2 *a* and *c*), within each group of peaks there were nevertheless several compounds with the same molecular weight. Identification of these analytes, such as the pair of unresolved compounds Gly^{Me}/X/Asp and Ser/X/Val (Fig. 2*b*), required the use of MS/MS analysis as a third dimension. The collision-induced dissociation spectrum of coeluting isobaric compounds (Fig. 3) contained fragments characteristic of the corresponding building blocks and of the core molecule itself (for the mechanism of daughter ion formation, see ref. 13). The fragment ions 1, 2, 3, and 4 (Fig. 3) were formed due to cleavage of the peptide bond and loss of one amino acid from the molecule (loss of Gly^{Me}, Asp, Ser, and Val, respectively). These daughter ions fragmented further with loss of the carboxyl terminus to yield the corresponding fragments 1′, 2′, 3′, and 4′. Fragment 4″ resulted from a further characteristic loss of H_2O from Ser. Thus, the pair Gly^{Me}/X/Asp and Ser/X/Val was resolved by identification of their daughter ions.

It was impossible to distinguish between compounds with Ile and Leu building blocks, because these amino acids had identical collision-induced dissociation spectra at the low-energy collision conditions essential to a triple quadrupole instrument. However, investigation of smaller libraries synthesized with Leu and Ile showed that these amino acids possessed

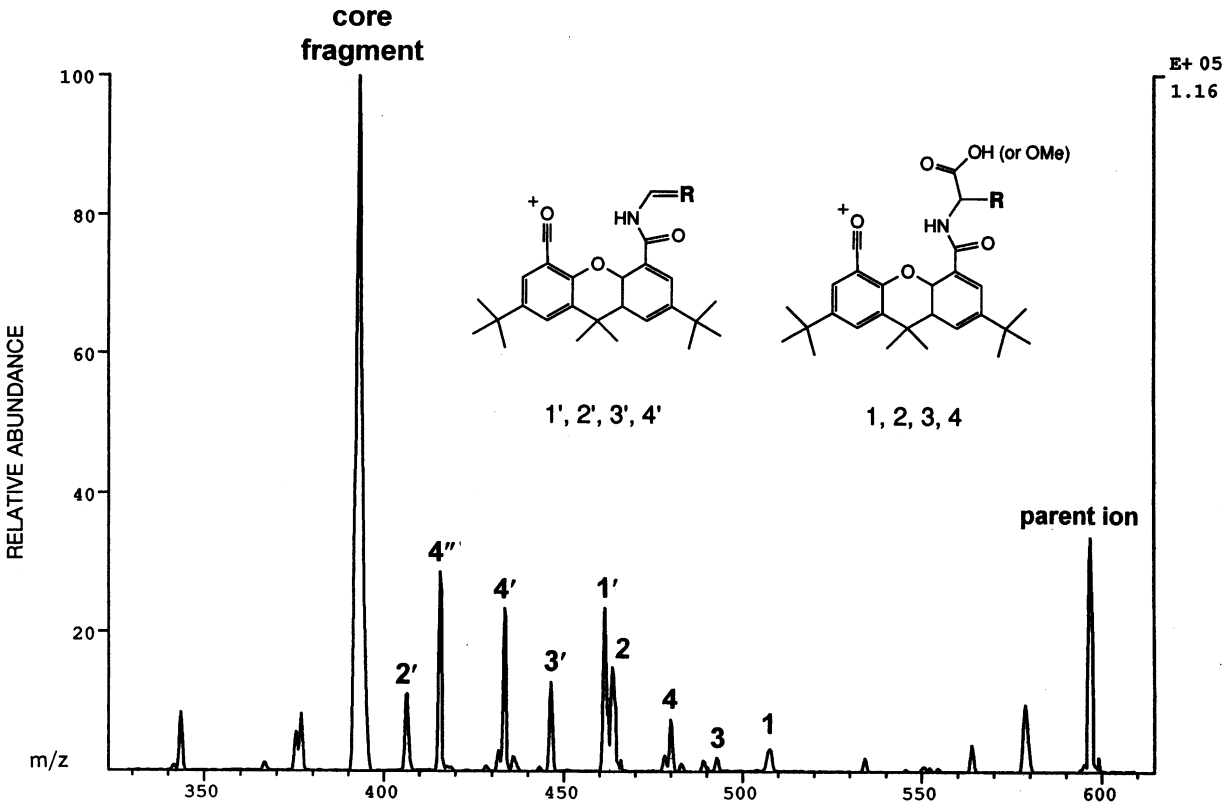


FIG. 3. Collision-induced dissociation spectrum of the peak corresponding to Gly^{Me}/X/Asp and Ser/X/Val (parent ion m/z = 597.5). Fragment ions characteristic to the xanthene derivatives containing Asp (**1**, **1'**), Gly^{Me} (**2**, **2'**), Val (**3**, **3'**), and Ser (**4**, **4'**, **4''**) are shown.

very close reactivity, such that derivatives with the Leu building block were present at nearly the same level as derivatives with the Ile building block. These results gave us ground to consider both Leu and Ile compounds present whenever the common molecular ion peak was identified by CE–MS/MS experiment. Two other pairs of building blocks, Ala and Gly^{Me}, and Asp and Thr^{Me}, also had identical molecular weights, but in each case these building blocks had different charges. Thus, unlike derivatives with Leu and Ile, derivatives with these amino acids were well separated from each other by CE, obviating the need for MS/MS (compare Ala/X/Leu and Gly/X/Leu in Fig. 2*a* and Thr^{Me}/X/Ala and Asp/X/Ala in Fig. 2*b*).

Analysis of representative xanthene derivatives as well as small mixtures of derivatives was performed to establish their relative ionization efficiencies (13). It was determined that in equimolar mixtures, the response ratios of the most sensitive to the least sensitive analogs (e.g., lysine- to valine-containing compounds) was not greater than 10:1. Based on this information, criteria for the presence or absence of the 171 expected library constituents were established. To be considered present, a compound had to appear at a concentration of no less than 1/10 of the most prominent derivatives in the library; thus, to be counted, Val/X/Val type compounds had to generate an ion trace with a peak area of at least 1% of the lysine-containing derivatives in the CE–MS electropherogram.

Results of the analysis of library A (Scheme **II**) are presented in the chart in Fig. 4. The chart shows that the majority of the expected compounds were formed under the reaction conditions employed (160 of 171). The derivatives not detected were those containing the Trp^{Me} building block in combination with neutral amino acids. This is likely the result of acidic degradation of the tryptophan side chain during the trifluoroacetic acid deprotection step. Another factor influencing the amount of these derivatives present in the final mixture was precipitation with ether/*n*-hexane, because compounds containing amino acid methyl esters precipitated to a lesser degree. Probably for the same reason, the Tyr^{Me}/X/Tyr^{Me}, Tyr^{Me}/X/Gly^{Me}, and Gly^{Me}/X/Gly^{Me} peaks were close to the level of the set threshold.

Evaluation of the final precipitation step was performed by analyzing the mixture synthesized from core **3** and the same set of 18 amino acids (library B, Scheme **II**). The compounds of this library were less hydrophobic, because two hydrogens in positions 2 and 7 were substituted for two *tert*-butyl groups. The CE–MS electropherogram of the mixture is shown in Fig. 5. The lower molecular weights resulted in an increased

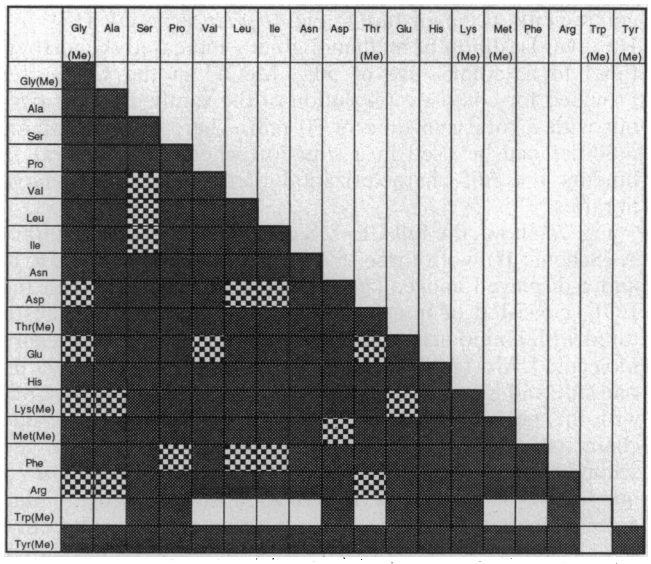


FIG. 4. Results of the mass spectrometric analysis of library A. Gray, detected by CE–MS; checkered, detected by CE–MS/MS; white, absent from the mixture.

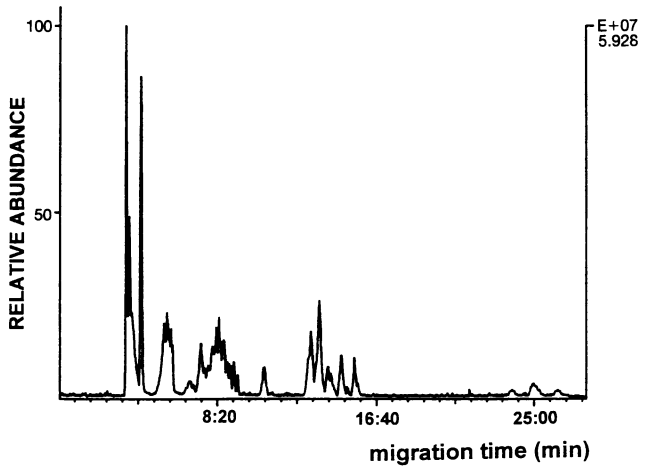


FIG. 5. CE–MS electropherogram of library B.

electrophoretic mobility of the analytes and, thus, longer migration times of negatively charged species compared to those of the components of the previous library. The separation efficiency was higher for this system, reducing the overall number of required MS/MS experiments for identification of the compounds. For example, derivatives with Lys^{Me} and another amino acid methyl ester eluted first and were separated from the other Lys^{Me}-containing compounds. Three compounds unresolved in the *tert*-butyl-xanthene library, Asp/X/Asp, Asp/X/Glu, and Glu/X/Glu (the last peak on Fig. 2*d*) were baseline-resolved (Fig. 5), and the ratio of the areas of the resolved peaks was 1:2:1. This corresponded to the theoretical molar ratio expected for these compounds, confirming that this combinatorial method is valid even with a large set of building blocks.

In general, the results of the analysis of library B correlated with the results for mixture A. The analytes were detected at the same level with insignificant variations. Again, most of the Trp^{Me}-containing compounds were not observed. The relative concentration of derivatives with Tyr^{Me} and Gly^{Me} building blocks was higher with core **1**, but still close to the threshold.

Some impurities, identified through MS/MS experiments, were found in both library A and library B. For example, the first peak in Fig. 2*a* corresponded to the isotope peak from a xanthene derivative substituted with Lys^{Me} and *N*-methyl-*N*-ethylamine (an impurity of the triethylamine used in synthesis). Another side-product, xanthene monohistidine monoacid (shown in Fig. 1*a*), was also present in the final library. In the mass range of the xanthene derivatives, there were only 12 peaks that could not be assigned to one of the expected library components. Most of these side-products were found to result from impure triethylamine as above or from the breakdown of certain amino acid side chains.

DISCUSSION

The results presented above demonstrate the potential of CE–MS/MS for the characterization of small-molecule libraries. Using CE–MS/MS, we were able to analyze complex mixtures A and B within a short time (≈20 min for each run). Detection of 160 of 171 expected xanthene derivatives (>90%) confirmed that the synthetic method of Scheme **II** is valid and allowed us to determine which building blocks should be avoided in future synthesis (in this case, tryptophan methyl ester). Because libraries A and B (Scheme **II**) examined the combinatorial chemistry of the closely spaced xanthene 4 and 5 positions, we have every reason to believe that combination of xanthene **2** and 18 amino acid building blocks (Scheme **III**) will generate a very large library of tetrasubstituted xanthenes, with at least 90% of the theoretically 52,650 compounds present and a minimum of side-products.

Capillary electrophoresis provided sufficient separation efficiency to resolve all analytes in libraries A and B by MS or MS/MS. Whereas 124 of the theoretically 171 derivatives overlapped in molecular weight, most of the 124 isobaric compounds were separated by CE; only eight MS/MS experiments were necessary for the identification of the 19 remaining molecules with overlapping molecular weights.

It was shown that even better separation of the library components could be achieved by adding an organic modifier such as MeOH to the CE buffer (see Fig. 1*c*), although this increased experiment time due to added library retention. Addition of 40% MeOH to the buffer allowed the resolution of most compounds with only four CE–MS/MS runs for 9 isobaric molecules, demonstrating the potential applicability of the technique to even larger libraries. In addition to the latter approach, other options may include the use of a coated capillary; this would allow detection of positively and negatively charged species in two different runs, increasing the separation efficiency of similarly charged analytes. Taking into account such possible improvements, and in view of the results achieved herein, we expect that CE–MS/MS will play a significant role in the growing field of combinatorial chemistry.

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