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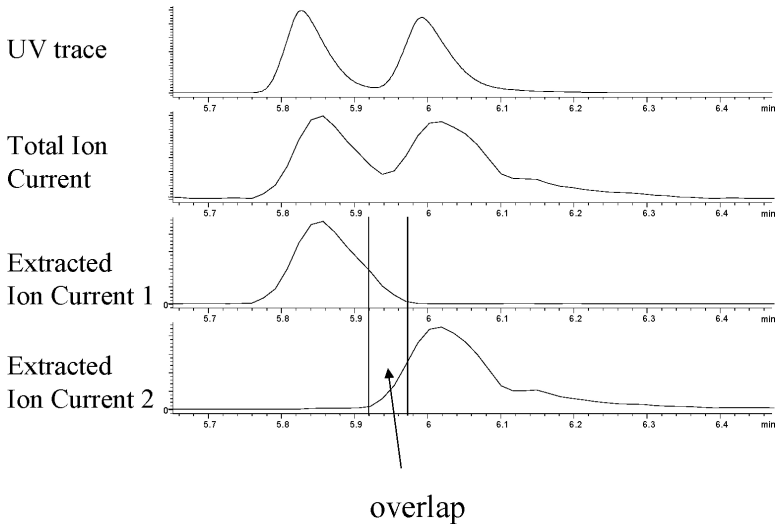

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Articles

Optimal Fraction Collecting in Preparative LC/MS

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In this paper, we discuss various methods for fraction collection in high-throughput chromatography. UV-triggered fractionation allows precise cutting of peaks. However, valuable fraction collector space is wasted, because many undesired compounds are collected. In mass-triggered fraction collection, the collector space is used more efficiently, because only peaks containing the desired products are collected. Unfortunately, mass peaks are broader than UV peaks, and therefore, fractions contaminated by a closely following peak are often collected. This can be avoided if the collection in preparative LC/MS occurs by a logical AND combination of UV- and mass-triggered collection. The success of this optimal collection mode is shown for three examples.

Introduction

One of the tasks of medicinal chemistry in drug research is the synthesis of large numbers of test compounds for high throughput screening. Most of the compounds are prepared by combinatorial chemistry on solid phase or, increasingly, in liquid phase. Typically, the compounds are submitted to screening after a purity check by LC/MS. However, increasing demands on the purity of test compounds have given rise to the application of automated high throughput purification methods. The high performance and broad applicability of reversed-phase chromatography has led to the development of fully automated preparative liquid chromatography systems capable of purifying 250 compounds/day.[1−9] Figure 1 depicts the typical setup of a preparative LC/MS system. The collection of a fraction can be triggered by either the UV or the mass detector. However, experience has shown that often purifications performed under standardized chromatographic conditions are not always successful. Synthetic byproducts or the starting materials may have physicochemical properties that are similar to the desired product. This leads to compound mixtures, which are difficult to separate without prior optimization of the chromatographic process. Aside from compounds not separated by the HPLC column, many unsuccessful purifications result from deficiencies in

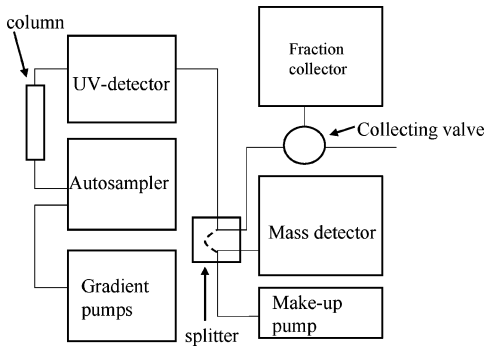

Figure 1. General setup of a preparative LC/MS system.

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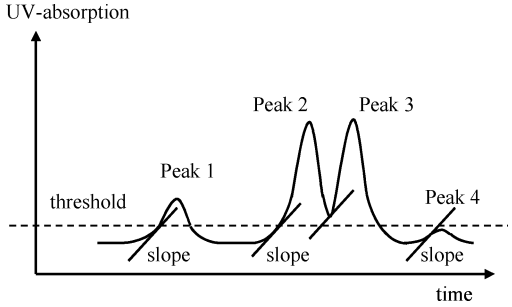

Figure 2. Collection by threshold or slope.

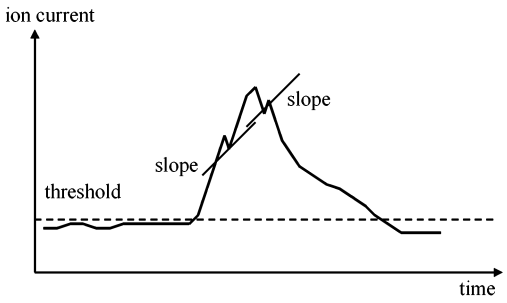

Figure 3. Mass peaks are often irregularly formed, and spikes can trigger fraction collection, if a slope method is used.

the fraction collecting process. Specifically, mass-triggered fraction collection often results in fractions contaminated by a closely following peak, although the corresponding UV trace shows almost baseline-separated peaks. The larger peak width of mass peaks as compared to UV peaks is responsible for this loss in separation power. In this report, various methods for the triggering of fraction collection are discussed, and a solution for this deficiency of mass-triggered fraction collection is presented.

Results and Discussion

UV-Triggered Fraction Collection. In this case, the collection valve of the fraction collector is triggered if the UV absorption exceeds a preset threshold value or the slope of the UV signal trace indicates the beginning of a peak (Figure 2). The closing of the collection valve is actuated if the respective signal falls below the threshold or the slope falls back below a specified value.

Figure 2 shows the disadvantages of the two collecting modes: if the collection is triggered by threshold only, peaks 2 and 3 will be collected in the same fraction vessel. Though the peaks are not baseline-separated, collecting them in different fraction vessels would likely give compounds of sufficient purity. Of course, a higher threshold would allow the collection of peaks 2 and 3 in different vessels; however, this would also lead to lower recoveries, because large portions of a peak are not collected. In addition, it is not feasible in high-throughput chromatography to optimize the threshold for every separation. Collection triggered by slope would collect peaks 2 and 3 in different collecting vessels, but in this case, the uninterestingly small peak 4 would also be collected. Practical experience shows that collection by slope will lead to the collection of numerous small side products that will rapidly fill up valuable fraction collector space.

The combination of both methods gives the best results; only peaks above the preset threshold are collected if the slope indicates the beginning or ending of a peak. Peaks 2 and 3 are collected in two different collecting vessels, and peak 4 is ignored.

Mass-Triggered Fraction Collection. In principle, mass-triggered fraction collection works like UV-triggered fraction collection: if the extracted ion current (EIC) of the target ion exceeds a predefined threshold, the collection of a fraction begins. If the EIC falls below the threshold, the collection stops. Collection by slope is not advisable because of the often irregular, jagged shape of mass peaks (Figure 3). Thus, the spikes in the upslope or downslope portion of the mass peak can trigger the opening and closing of the collection valve several times. As a consequence, a single peak would be collected in several collection vessels.

Because the majority of compounds ionize better in the positive ion mode than in the negative ion mode, mass-triggered fractionation usually is performed in the positive mode. However, frequently compounds fragment in the positive ion mode so strongly that collection of these compounds is not possible. In these cases, fractionation in the negative ion mode often gives better results. In high-throughput chromatography, it is not feasible to test the optimal ionization mode for every separation; therefore, switching between the positive and the negative ion mode during the separation will most reliably collect all sorts of compounds.

UV- and Mass-Triggered Fraction Collection in Comparison. With UV-triggered collection, all peaks that fulfill the threshold and slope conditions are collected; therefore, valuable space on the fraction collector is filled up with many undesired compounds. Consequently, the desired compounds have to be found among the many unwanted ones, typically through analysis of each fraction by flow injection mass spectrometry. Then the proper fractions have to be "cherry-picked" from the undesired ones. Theoretically, mass-triggered fraction collection should be superior to UV-triggered collection: only one fraction containing the desired product should be collected, no matter how many other compounds the separated sample contains.

In contrast, the advantage of UV-triggered collection compared to mass-triggered collection is the smaller UV peak width.[10] Mass peaks are up to 50% broader than UV peaks in preparative LC/MS. This broadening of mass peaks is easily overlooked if the UV detector is not installed in the preparative flow of the splitter. If the UV detector is placed in the flow directed toward the mass detector, some peak broadening is caused by the splitter, and UV peaks appear almost as broad as the corresponding mass peaks. The inertia of the mass detector (dead volumes, scanning rate, etc.) and the influence of the splitter are responsible for this peak broadening. This broadening of mass peaks is responsible for contaminated fractions if the collected peak is closely followed by another peak. An example of this phenomenon can be seen in Figure 4. The UV trace in Figure 4 shows two almost baseline-separated peaks, whereas the corresponding total ion current trace (TIC) shows two overlapping peaks. This overlap can be seen clearly in the extracted ion current traces. If the first peak is collected by mass triggering

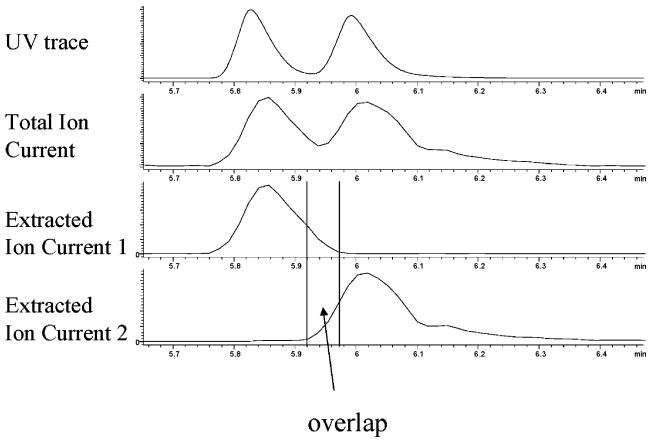

Figure 4. Comparison of UV and MS peaks. The TIC mass peaks and the extracted ion peaks are ~50% broader than the corresponding UV peaks.

at a low threshold, the resulting fraction would be contaminated by the second peak. At higher thresholds, it is possible to collect a pure fraction, but only by accepting a lower recovery. Furthermore, compounds that do not ionize well might not be collected at all at high thresholds. Another option for obtaining pure fractions is to collect a single peak in several fractions ("heart cutting") and to retain only the fractions containing pure product. The disadvantage of this procedure is again lower recovery and inefficient use of fraction collector space.

So, the dilemma of mass-triggered fraction collection is either accept poor recoveries or else risk collecting too many impure compounds. Practice shows that up to 20% of the compounds collected by mass-triggered fraction collection have to be discarded because impurities are too high. The solution to this dilemma is obvious. The collection valve should be triggered by the UV method, but only in the presence of the target ion of the desired compounds. Then the full separation power of the HPLC column can be exploited without wasting valuable fraction collector space.

In the following three examples, the results obtained with the combination of fraction triggering by UV and mass are shown.

Example 1. The separation of compound **1** out of a mixture of compound **1** and **2** (for structures, see Figure 8) by mass-triggered fractionation in the positive ion mode gave a fraction of **1** contaminated strongly by compound **2**. The vertical lines in Figure 5a indicating the beginning and end of a fraction show that both peaks were collected into the same fraction vessel. Essentially the same result was obtained by mass-triggered fractionation in the negative ion mode: the vertical lines in Figure 5b show that a large portion of the second peak was collected in the same fraction as the first peak. Reanalysis of the collected fractions by analytical HPLC showed that the fractions contain 52 and 41 area % of compound **2**, respectively. Fraction triggering by a logical AND combination of UV and mass, however, resulted in a fraction containing pure compound **1**. The vertical lines in Figure 5c show the end of the first fraction at the end of the first peak. A second fraction was collected, because the slope of the UV trace rises again, and the target ion of the desired

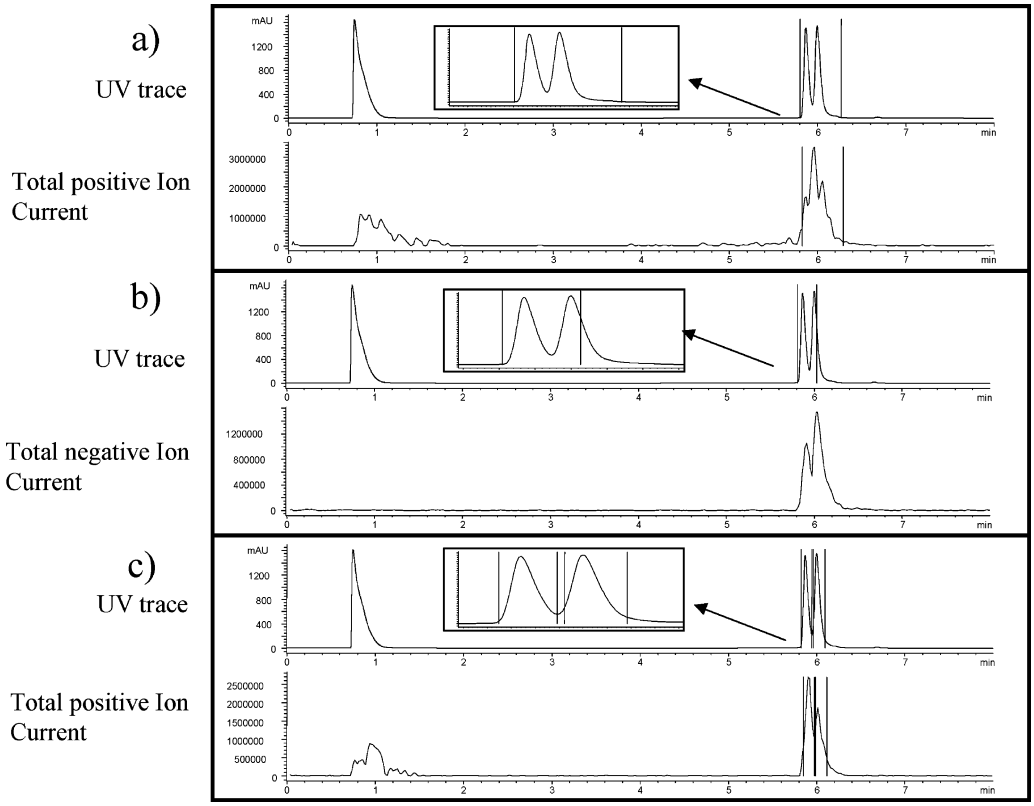

Figure 5. Separation of compounds **1** and **2** by different fraction collecting methods: (a) by mass-triggered collection in positive ion mode, (b) by mass-triggered collection in negative ion mode, (c) by the combination of UV- and mass-triggered fraction collection. A 10-mg portion of each compound was dissolved in 500 μL of DMSO and injected. The chromatographic conditions described in the Experimental Section were applied.

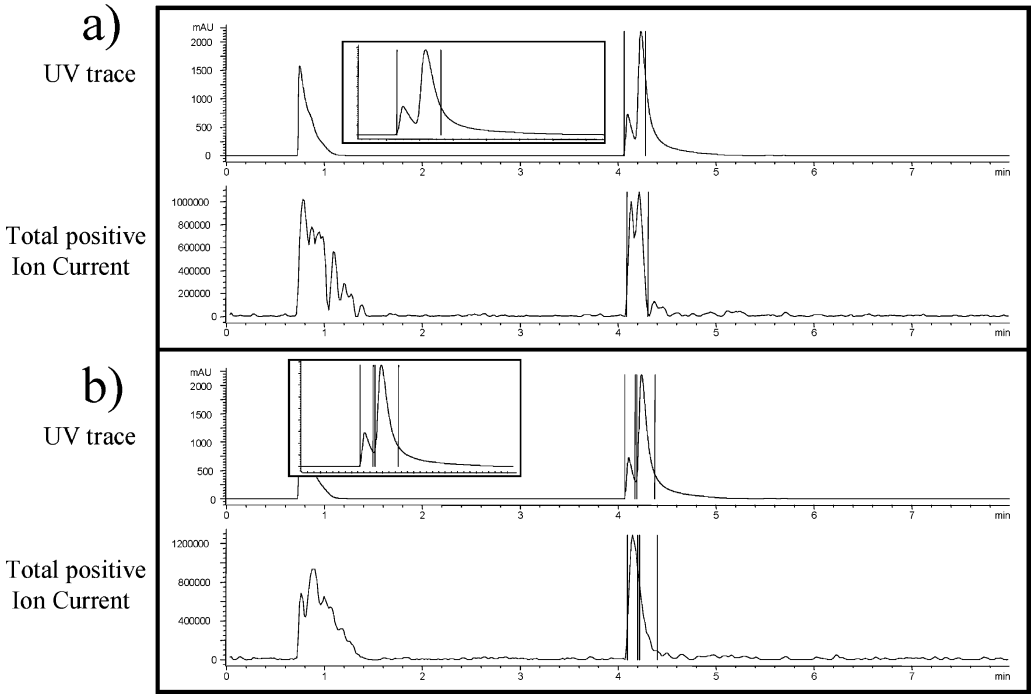

Figure 6. Separation of compounds **3** and **4** by different fraction collecting methods: (a) by mass-triggered collection in positive ion mode, (b) by the combination of UV- and mass-triggered fraction collection. A 10-mg portion of each compound was dissolved in 500 μL of DMSO and injected. The chromatographic conditions described in the Experimental Section were applied.

product was still present. Reanalysis of the first fraction showed 97 area % of compound **1**.

Example 2. The separation of compound **3** out of a mixture of compound **3** and **4** (structures see Figure 8) by mass-triggered fractionation in the positive ion mode gave a fraction of **3** contaminated strongly by compound **4** (see Figure 6a). Reanalysis of the collected fraction by analytical HPLC showed a 47.9 area % contamination by **4**. Fraction triggering by a logical AND combination of UV and mass, however, resulted in a fraction of pure compound **3** (see Figure 6b). Reanalysis of the first fraction showed 96.3 area % of compound **3** and 3 area % of **4**. This result is remarkable, because the two peaks were barely resolved.

Example 3. The separation of compound **1** (the second peak) out of a mixture of compounds **1**, **5**, and **6** (structures see Figure 8) by mass-triggered fractionation in the positive ion mode gave a fraction of **1** almost uncontaminated by compound **6** (see Figure 7a). Reanalysis of the collected fraction by analytical HPLC showed a 0.4 area % contamination by **6**. In this case, the ionization of the second peak was incidentally suppressed by the appearance of the ions of the third peak. Mass-triggered fractionation in the negative ion mode, however, gave a different result (see Figure 7b). Now the fraction containing **1** was strongly contaminated by **6**. Reanalysis of the collected fraction by analytical HPLC showed that the fraction contains 28 area % of compound **6**. Fraction triggering by a logical AND combination of UV and mass resulted again in a fraction of pure compound **1** (see Figure 7c). Reanalysis of the collected fraction showed only 0.8 area % of **6**.

Conclusion

The optimal method of fraction collection in preparative LC/MS is the logical AND combination of UV- and mass-triggered collection. The collection valve should be triggered by a UV slope method, but only in the presence of the target ion of the desired compounds. This method combines the advantages of the two collection modes and avoids their disadvantages.

The advantage of UV-triggered fractionation is the precise cutting of peaks. The disadvantage is the collection of all peaks, thereby wasting valuable collector space. The advantage of mass-triggered fractionation is the efficient use of collector space, because only peaks containing the desired products are collected. The disadvantage is that mass peaks are broader than UV peaks. Therefore, fractions contaminated by a closely following peak are often collected. The advantages of both methods can be combined, if the collection valve is triggered by the UV method, but only in the presence of the target ion of the desired compounds. Then, the full separation power of the HPLC column can be exploited without wasting valuable fraction collector space. The benefits of the logical AND combination of UV- and mass-triggered collection are clearly demonstrated in three experimental examples.

Experimental Section

Hardware Configuration. The experiments were performed with the Agilent 1100 Series Purification System, set up as shown in Figure 1. Preparative flow was generated with a pair of Agilent 1100 series preparative pumps (flow rates up to 100 mL/min). The makeup pump was an Agilent 1100 series isocratic pump. The Agilent 1100 series preparative autosampler and fraction collector PS were used. The UV detector was an Agilent 1100 series diode array detector with a preparative flow cell (0.06 mm path length). The split of the preparative flow was accomplished by the Agilent

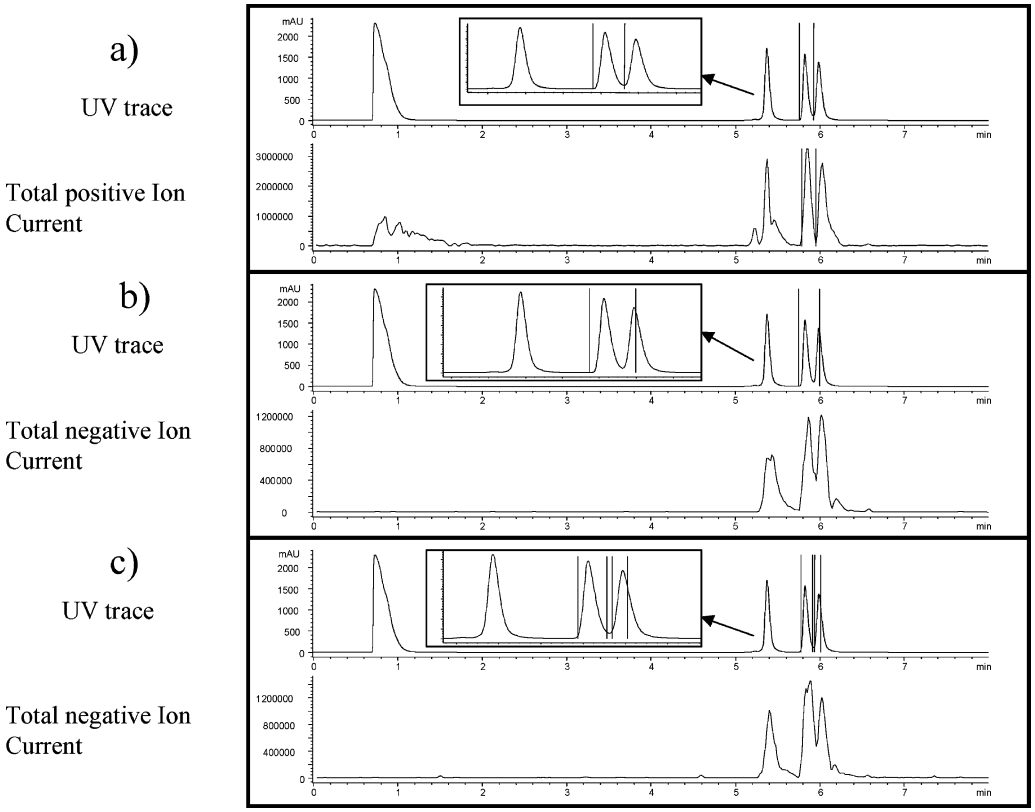

Figure 7. Separation of compounds **1**, **5**, and **6** by different fraction collecting methods: (a) by mass-triggered collection in positive ion mode, (b) by the combination of UV- and mass-triggered collection in negative ion mode, (c) by the combination UV- and mass-triggered fraction collection. A 10-mg portion of each compound was dissolved in 500 μL of DMSO and injected. The chromatographic conditions described in the Experimental Section were applied.

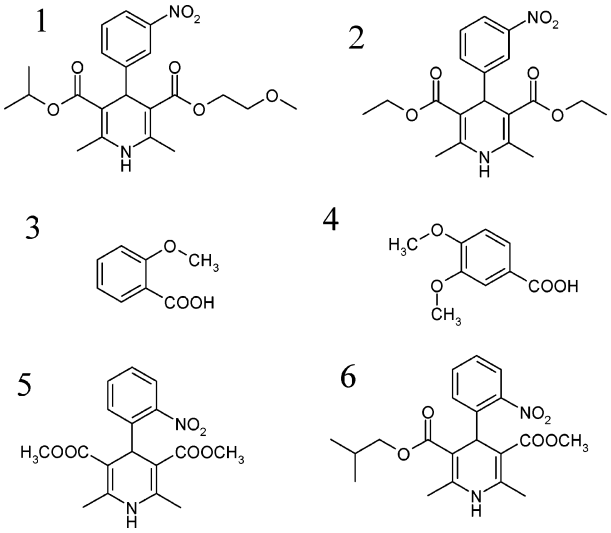

Figure 8. Structures of compound **1**−**6**.

Active splitter set to a split ratio of 1:1000. The mass spectrometer employed was an Agilent 1100 LC/MSD SL system. The LC/MS system was controlled by Agilent ChemStation and the Purification software.

Fraction Collector Settings. Fraction collection was performed using a logical AND connection of peak-based fraction collection on the UV signal- and mass-based fraction collection. Fraction triggering on the UV signal was done on the basis of slope only. Settings for both the up and down slope were 75 mAU/s, respectively. Mass-based fraction collection was performed on threshold only (slope 0 counts/s) with a threshold setting of 100 000 counts.

HPLC Conditions. For all preparative separations, a 21 × 50-mm biaxial compression column from Macherey-Nagel (VP 50/21 Nucleosil 100-5 C18 Nautilus) with a precolumn from Grom (GROM-SIL 120 ODS-4 HE, 15 μ, 10 × 20 mm) was used. The following gradient method was applied: A water, B acetonitrile; 10% B hold for 2.0 min; 10−90% B in 4.0 min; 90% B hold for 1.0 min. The flow rate was 25 mL/min; the detector wavelength, 220 nm; and the column temperature was ambient.

For the reanalysis of the fractions collected in examples 1 and 3, a 4.6 × 75-mm Zorbax SB-C18 column (3.5 μm) was used. The following gradient method was applied: A water, B acetonitrile; 40−78% B in 6 min. The flow rate was 1 mL/min; the injection volume, 5 μL; the detector wavelength, 220 nm (bandwidth 8 nm); and the column temperature was 25 °C. For the reanalysis of the fractions collected in example 2, a 2 × 125-mm column from Macherey-Nagel (VP 50/21 Nucleosil 120-5 C18 Nautilus) was used. The following gradient method was applied: A water containing 0.05% phosphoric acid, B acetonitrile; 5% B hold for 1.0 min; 5−30% B in 3.0 min; 30% B hold for 2.5 min. The flow rate was 1.0 mL/min; the injection volume, 3 μL; the detector wavelength, 214 nm; and the column temperature was 40 °C.

Mass Spectrometry Conditions. Mass spectra were acquired either in the positive or negative ion mode scanning or by switching from the positive to the negative ion mode

(each 50% of the scan time) over the mass range of 100−500. The following ion source parameters were used: drying gas flow, 13.0 L/min; nebulizer pressure, 45 psig; drying gas temp, 350 °C; capillary voltage, 3000 V for positive and negative mode. Makeup flow was 0.8 mL/min, and the makeup solvent was methanol with 0.1% formic acid.

Compounds. Compounds **3**, **4**, and **5** are commercially available. Compounds **1**, **2**, and **6** can be prepared by literature procedures.[11,12]

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