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Analytical tools for the detection and characterization of biologically active compounds from nature

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Abstract

Nature has been recognized as a rich source of medicinal compounds for hundreds to thousands of years. Today, a vast range of drugs, which represent the cornerstones of modern pharmaceutical care, are either natural products or have been derived from them. Though providing high chemical diversity, the implementation of natural product research in modern High Throughput Screening (HTS) programs has decreased considerably during the last decade. Instead, seemingly more cost-effective and HTS compatible approaches such as combinatorial chemistry have been explored in an effort to increase the number and diversity of chemical entities. However, pharmaceutical companies are facing enormous challenges as advances in human genome description have led to an increasing number of new molecular drug targets and intensified the need for new, additional molecular diversity as a source of novel drug molecules. Natural products still offer an attractive route to alternative chemical diversities and possess a proven track record in pharmaceutical medications. Consequently, a high demand exists for novel and highly efficient screening technologies, which enable successful incorporation of natural products in drug discovery programs. Over recent years several techniques have been developed, which interfaced continuous-flow biochemical detection with a range of analytical instruments, such as LC, DAD, UV and MS. The combination of analytical technologies and continuous-flow biochemical detection has enabled biological and chemical evaluation of bioactive molecules within a single analysis and profoundly reduces the time required for compound characterization. Recent advances in this field as well as the application of continuous-flow biochemical detection for the screening of complex mixtures, such as natural product extracts, are reviewed in this paper.

Introduction

It is estimated that about one third of currently marketed drugs are related to natural products (Onaga, 2001; Grabley and Thiericke, 1999). Indeed, nature has been known as an abundant source of medicinal molecules for thousands of years. Numerous examples are known from medicine, which demonstrate the innovative potential of natural compounds and underline the high chemical diversity present in nature (Strege, 1999). Biologically active compounds have been isolated from a wide variety of natural sources, ranging from marine organisms, such as sponges (Rashid et al., 2002; Schwartsmann et al., 2001) and sea cucumbers (Maier et al., 2001; Li, 2000) to microbes (Strobel et al., 2002). Recently discovered drugs from natural products include the powerful anti-tumor drug taxol, which was first isolated from the branches of *Taxus brevifolia* (Taxaceae) (Foa et al., 1994; Huizing et al., 1995), the immunosupressants cyclosporine A and FK506 (Ruhlmann and Nordheim, 1997; Gold, 1997), and lovastatin (Illingworth, 2001). These compounds play a profound role in modern pharmaceutical care. Currently, many natural product studies are ongoing and are focused on discovering drugs for a range of therapeutic areas. The recent discovery of anti-proliferative compounds in a range of marine organisms, such as ecteinascidine (ET-743), aplidine and kahalalide F, is illustrative hereof (Jimeno,

2002). In the past, the pharmaceutical industry has frequently implemented natural product extracts in drug discovery screening programs. However, despite the high chemical diversity provided by nature, the obstacles encountered in natural product screening, combined with the advent of new technologies, such as High Throughput Screening (HTS) and combinatorial chemistry, caused a fundamental paradigm shift in drug discovery. During the last decade many pharmaceutical companies abandoned natural product extracts and incorporated other sources of chemical diversity, such as synthetic compound libraries, instead. Synthetic compound libraries reduce many problems associated with natural product screening, including false positives, recurring bioactive molecules, autofluorescence and the time-consuming identification of active compounds. Moreover, pure compound samples generally show high compatibility with HTS, thus increasing screening efficiency as a result. Nowadays, throughput numbers of 10,000 to 100,000 samples/day are achieved with state of the art HTS platforms. However, despite the paradigm shift towards more cost-effective approaches, chemical diversity of synthetic compound libraries has generally been considered significantly less compared to that offered by natural products (Strege, 1999). As chemically diverse compound libraries represent a crucial requirement for the discovery of lead molecules, a need exists for new, additional molecular diversity as a source of novel drug molecules. This need has been intensified by the recent progress in human genome description. During the last years, a multitude of new molecular drug targets has been identified and has provided the pharmaceutical industry with a major challenge. As a result, several strategies have recently been developed in order to improve the chances of finding promising lead molecules. Chemical genomics and combinatorial synthesis of natural product libraries represent two of these newly emerging technologies that focus on creating more advanced and chemically diverse compound libraries. In an increasing number of genomics approaches, information-enriched libraries, containing chemically diverse small molecules, are used in an attempt to understand global functioning of proteins and derive lead structures from the acquired information, a process known as chemical genomics (Hacksell et al., 2002; Zanders et al., 2002; Goodnow, 2001; Darvas et al., 2001; Zheng and Chan, 2002; Watterson et al., 2002a, b; Chan et al., 2000; Willson et al., 2001; Weber, 2000; Vidal and Endoh, 1999). In addition, combinatorial chemistry has adopted natural scaffolds as chemical building blocks in order to generate compound libraries with enhanced chemical diversity and to support lead optimization (Nielsen, 2002; Eldridge et al., 2002; Abel et al., 2002; Arya, 2002; Breinbauer et al., 2002; Hall et al., 2001; Bindseil et al., 2001; Wipf et al., 2000). Compound library design represents one strategy to accelerate detection of lead compounds. Alternatively, efficient technologies enabling cost-effective screening of additional chemically diverse samples, such as natural products, are newly emerging and potentially powerful techniques to detect novel drug-like molecules.

On-line liquid chromatography – biochemical detection (LC-BCD)

Nowadays, the number of samples identified and the timescales dictated by modern HTS programs require rapid and effective prioritization of extracts. The ability to rapidly identify known or undesirable compounds in natural product extract libraries is a critical step in an efficiently run natural products discovery program. This process, commonly called dereplication, is important to prevent the unnecessary use of resources on the isolation of compounds of little or no value for development from extracts used in the screening process. Resources can then be focused on samples containing the most promising leads. Dereplication strategies typically employ a combination of separation sciences, spectroscopic methods (Wolfender et al., 1998), and database searching (Corley and Durley, 1994) High performance liquid chromatography (HPLC) has been the most reliable tool for the separation of complex mixtures of small molecules. Reversed phase HPLC on octadecylsilane (ODS or C_{18}) is recognized as the most broadly applicable of bonded phases for this purpose. When interfaced with a diode array detector (DAD), HPLC allows an analyst to identify known compounds by comparison of their HPLC retention times and UV spectra. More recently, the advent of electrospray (ES) and atmospheric pressure chemical ionization (APCI) interfaces have provided mass spectrometry (MS) interfaces which are applicable to the analysis of a wide range of molecules and are compatible with liquid chromatography. In the past five years, LC-MS has become a widely used tool for the dereplication of natural products (Wolfender et al., 1995; Shigematsu, 1997) because the nominal molecular weight of a com-

pound can be used as a search query in nearly all databases.

Dereplication of the active component(s) in crude natural products extracts requires some form of feedback from the bioassay, which was initially used to detect the biological activity. This is necessary regardless of the separation technique and analytical method (DAD or MS) used. A common strategy has been to collect fractions from the HPLC separation in deep-dish microtiter plates or tubes and then re-submit the individual fractions to the original assay. This approach requires dessication of fractions to remove the HPLC solvents, which are usually incompatible with the bioassay, resuspending the fractions in a compatible solvent (water or DMSO), and then individual assaying of each fraction. This process is not cost effective being both time and labor intensive. Consequently, as a result of the increasing emphasis on the generation of new lead compounds, faster cycle times and high efficiency, many pharmaceutical companies have moved away from the natural products area. Currently, almost every large pharmaceutical company has established HTS infrastructures and possesses large combinatorial compound libraries, which cover a wide range of chemical diversity. However, the ability to detect the desired biological activity directly in the HPLC effluent stream and chemically characterize the bioactive compounds on-line would eliminate much of the time and labor taken in the fraction collection strategy. This way, cycle times, expenses and the isolation of known or undesirable compounds would be reduced dramatically, allowing natural products to be screened in an efficient and cost effective manner. This way, typical bottlenecks, which have been associated with natural product screening, are largely overcome.

Recently, such an on-line HPLC biochemical detection (LC-BCD) system has been described for a range of targets, such as the human estrogen receptor (hER) (Schobel et al., 2001; Oosterkamp et al., 1996a), the urokinase receptor (Oosterkamp et al., 1998), acetylcholinesterase (Ingkaninan et al., 2000; Ingkaninan et al., 1999) and phosphodiesterase (Schenk et al., 2003 in preparation). Figure 1 displays a schematic representation of such a LC-BCD system. After injection, the complex mixture is chromatographically separated (Figure 1A–B). Subsequently, the separated compounds are introduced in a biochemical assay (Figure 1C). In contrast to conventional microtiter-type bioassays, the interactions of the extracts and the biochemical reagents proceed at high speed in a closed continuous-flow reaction detection system. When sufficient resolution is achieved the individual contribution of the bioactive compounds to the total bioactivity is obtained within a single run. Moreover, by combining on-line biochemical detection with complementary chemical analysis techniques such as mass spectrometry, i.e. LC-BCD-MS (Figure 1D), chemical information, which is crucial for the characterization and identification of bioactive molecules, is obtained in real time. Biochemical responses are rapidly correlated to the recorded MS and MS/MS data, thus providing chemical information such as molecular weight and MS/MS fingerprint (Figure 1E–G).

Compared to traditional screening approaches of complex mixtures, which are often characterized by a repeating cycle of HPLC fractionation and biological screening, LC-BCD-MS analysis speeds up the dereplication process dramatically. Moreover, the technique enables drug discovery programs to access the enormous chemical diversity offered by complex mixtures as a source of novel drug like molecules.

On-line biochemical assays

Competitive bioassay formats

Continuous-flow biochemical assays represent key elements in LC-BCD. Over recent years a range of assay formats has been developed which enabled implementation of a large variety of protein targets. One of the most commonly used bioassay formats is based on the competition of fluorescence labeled ligands and biologically active molecules in the samples (Lutz et al., 1996, 1997; Oosterkamp et al. 1994a, b); 1996b, 1997. An example of such an approach is shown in Figure 2. In this case, ligands for the aspartic protease cathepsin D are detected by performing a two-step heterogeneous bioassay setup. During the first step of the bioassay, cathepsin D is added to the HPLC effluent. Subsequently, the mixture is allowed to interact for a determined period of time during which ligands, originating from the injected sample, bind to the target protein. In a second step, the remaining free binding sites on cathepsin D are titrated by adding a fluorescence labeled ligand, i.e., pepstatin BODIPY FL, to the reaction mixture. Binding of pepstatin BODIPY FL to cathepsin D reduces the free concentration of this fluorescent molecule. By introducing a restricted access column (RA-column) an efficient separation of affinity-bound and unbound molecules is obtained. The chromatographic material is

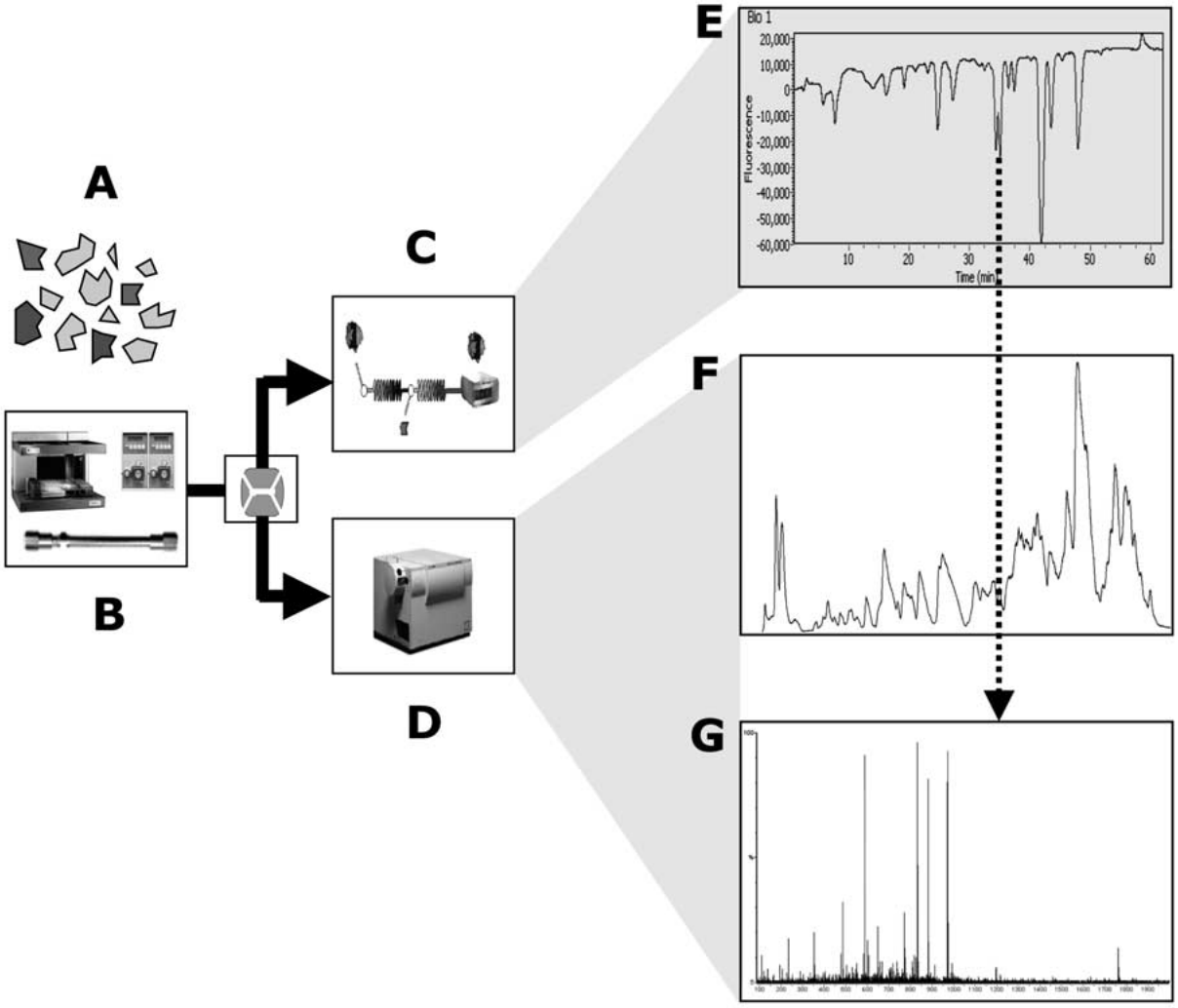

Figure 1. Schematic representation of on-line liquid chromatography – biochemical detection coupled to mass spectrometry. A. Injection of complex mixture. B. Separation of mixture by HPLC. C. Analysis of mixture by continuous-flow biochemical detection. D. Analysis of mixture by mass spectrometry. E. Output biochemical detection: bioaffinity chromatogram. F. Total ion current MS (TIC). G. Mass spectrum corresponding to response indicated in the bioaffinity chromatogram.

characterized by small hydrophobic pores (<14 kDa) and a surface coated with hydrophilic compounds. In case of the cathepsin D bioassay, unbound pepstatin BODIPY FL molecules are efficiently trapped in the hydrophobic pores of the RA-material. The more bulky target protein (45 kDa) and affinity complexes however do not penetrate the small pores and pass the RA-column almost unretained. The residence times of the affinity complexes in the RA-column are typically in the order of seconds, which reduces dissociation compared to other separation techniques, such as size exclusion chromatography (SEC). After separating unbound and affinity-bound pepstatin BODIPY FL the fluorescence intensity of the reaction mixture is measured continuously. The presence of ligands in the injected samples causes an increase in the free pepstatin BODIPY FL concentration as the number of free binding sites on cathepsin D is decreased temporarily. As a result, less fluorescent affinity complexes reach the detector, causing a decrease in fluorescence intensity. Consequently, cathepsin D inhibitors are observed as negative responses in the biochemical readout.

Several parameters play a crucial role in obtaining highly sensitive bioassays. In general, reaction kinetics is thoroughly investigated in optimization studies. This way optimum bioreagent concentrations and re-

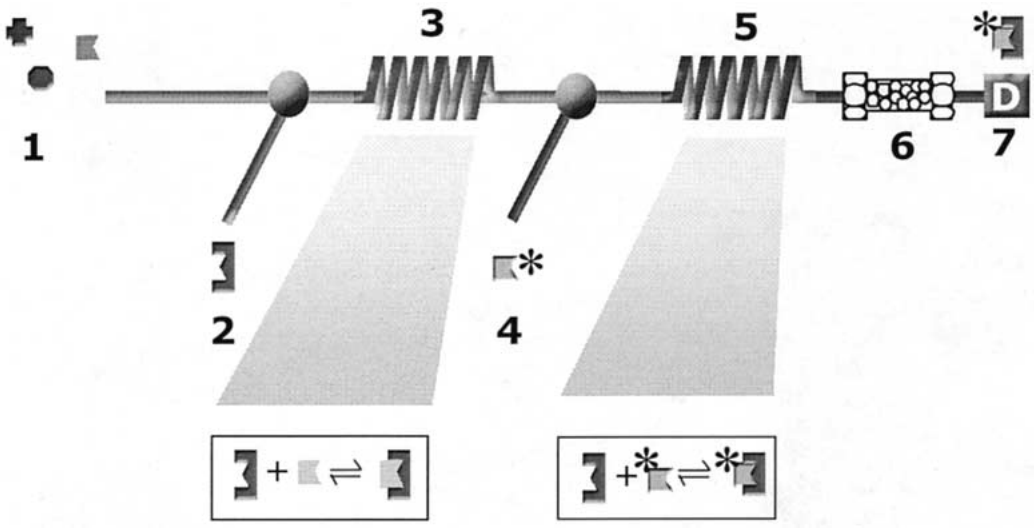

Figure 2. Schematic representation of a competitive bioassay format. 1. Injection of analytes. 2. Addition of cathepsin D (100 nM, 10 μl/min). 3. Reaction coil. 4. Addition of pepstatin-BODIPY FL (10 nM, 10 μl/min). 5. Reaction coil. 6. Restricted access column (ADS, C18, 2 * 5 mm). 7. Fluorescence detector.

action times for the biochemical assay are selected. In addition, non-specific binding of target proteins and reporter molecules, buffer composition and temperature represent typical parameters, which are optimized under continuous-flow conditions.

Substrate conversion based assay formats

Next to the competitive bioassay format, substrate conversion based approaches are usually highly suitable to be implemented in continuous-flow bioassays. Sufficient enzymatic activity however, is an important prerequisite for obtaining low detection limits. The general configuration of substrate conversion based bioassays is displayed in Figure 3. After separation of the analytes the enzyme is added to the carrier phase and is allowed to interact with the molecules for a determined period of time. During the second step of the assay the substrate is added to the reaction mixture. Typically, the substrates used are internally quenched and do not exhibit a high fluorescence quantum yield until enzymatically converted. The fluorescence enhancement, produced by the converted substrate molecules, allows these bioassay formats to be conducted homogeneously, i.e. no separation of reporter molecules is required to detect binding of ligands to the enzyme targets. In this case, ligands temporarily decrease the enzymatic conversion rate of the substrate, which reduces the fluorescence intensity. As a consequence, ligands are often detected as negative responses in the biochemical readout. Alternatively however, positive responses are observed when the fluorescence intensity of the substrate is decreased after conversion. In general, the bioassay format described here is applicable for a multitude of enzyme targets, including kinases, phosphatases, phosphodiesterases, angiotensin converting enzyme, caspases and cathepsins. The homogeneous nature of the method usually ensures robust assay performance during extended runs (20 h).

LC-BCD and natural product extract libraries

Estrogenic compounds in plant extracts

Recently, a homogeneous on-line bioassay system was developed for screening a large plant natural product library for estrogen receptor (ER), α and β, binding. During the last decade, interest in the pharmaceutical potential of phytoestrogens for the treatment of diseases like osteoporosis and breast and prostate cancer has increased dramatically. Phytoestrogenic compounds, which are commonly found in fruits, herbs and leguminous plants, have structural similarities with some mammalian estrogens and have been shown to exert weak estrogenic behavior (Kuiper et al., 1998). These food components are consumed in high quantities in cultures that show low incidence

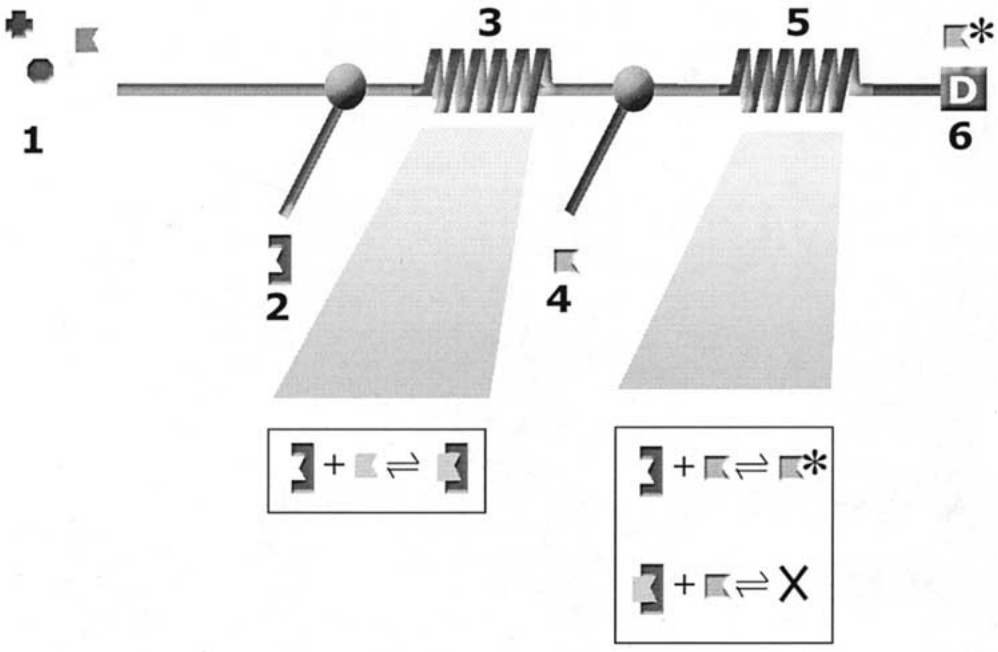

Figure 3. Schematic representation of a substrate conversion based bioassay format. 1. Injection of analytes. 2. Addition of enzyme. 3. Reaction coil. 4. Addition of internally quenched substrate. 5. Reaction coil. 6. Fluorescence detector.

of hormonally-dependent cancers, suggesting that they may act as chemopreventive agents (Setchell and Cassidy, 1999). The discovery of the human breast cancer cell growth inhibitory properties of the natural product resveratrol underlines these suggestions (Kurzer and Xu, 1997). In total approximately 10,000 extracts were first screened using the fast flow injection analysis where the chromatographic system is bypassed. In this way, bioactive samples are efficiently filtered out of the large library before processing them in the more time consuming HRS-MS mode.

The basic principle of the employed homogeneous BCD system is identical for both the flow injection and the LC-BCD-MS systems. In both configurations the ER is added to the sample, either in batch or continuous-flow mode and the mixture is allowed to react for a maximum of 30 sec. (Figure 4). In a second step, a fluorescent ligand (reporter molecule) is added to saturate the free binding sites of the ER, e.g., the fluorescent phytoestrogen coumestrol. Coumestrol is a high affinity ligand for ERs, exhibiting an affinity constant 20% relative to that reported for17β-estradiol (Lu and Serrero, 1999). It exhibits a blue fluorescence with a maximum at 438 nm when excited at 340 nm. When bound to the ER, the fluorescence emission of coumestrol shows a blue shift to 410 nm, while the fluorescence intensity of the ER-coumestrol complex is approximately four times higher than the fluorescence intensity of unbound coumestrol. The presence of estrogenic compounds in the test sample is thus detected as a reduced enhancement of the coumestrol fluorescence intensity at 410 nm.

Identification of bioactive extracts

The flow injection assay system described here was successfully utilized to screen a natural product extract library of 9888 extracts. 4032 extracts were analyzed at a 1:30 dilution, while the remaining 5856 samples were screened at a threefold higher dilution. The hit rate was 8.9% and 12.6% for the 1:30 (90% cut off) and 1:90 (70% cut off) dilutions, respectively. 49.0% (1:30 dilution) and 54.7% (1:90 dilution) of the identified hits could be confirmed, indicating that the results of the assay at the higher dilution were more reliable. The selectivity of the confirmed hits towards ERα and ERβ is shown in Figure 5. Here on the positive and negative y-axis ERα and ERβ selective hits, respectively, are displayed. A hit was defined as ER$\alpha(\beta)$ selective when the difference between the inhibition values was greater than 60%. As can be seen from Figure 5, six selective extracts could be identified.

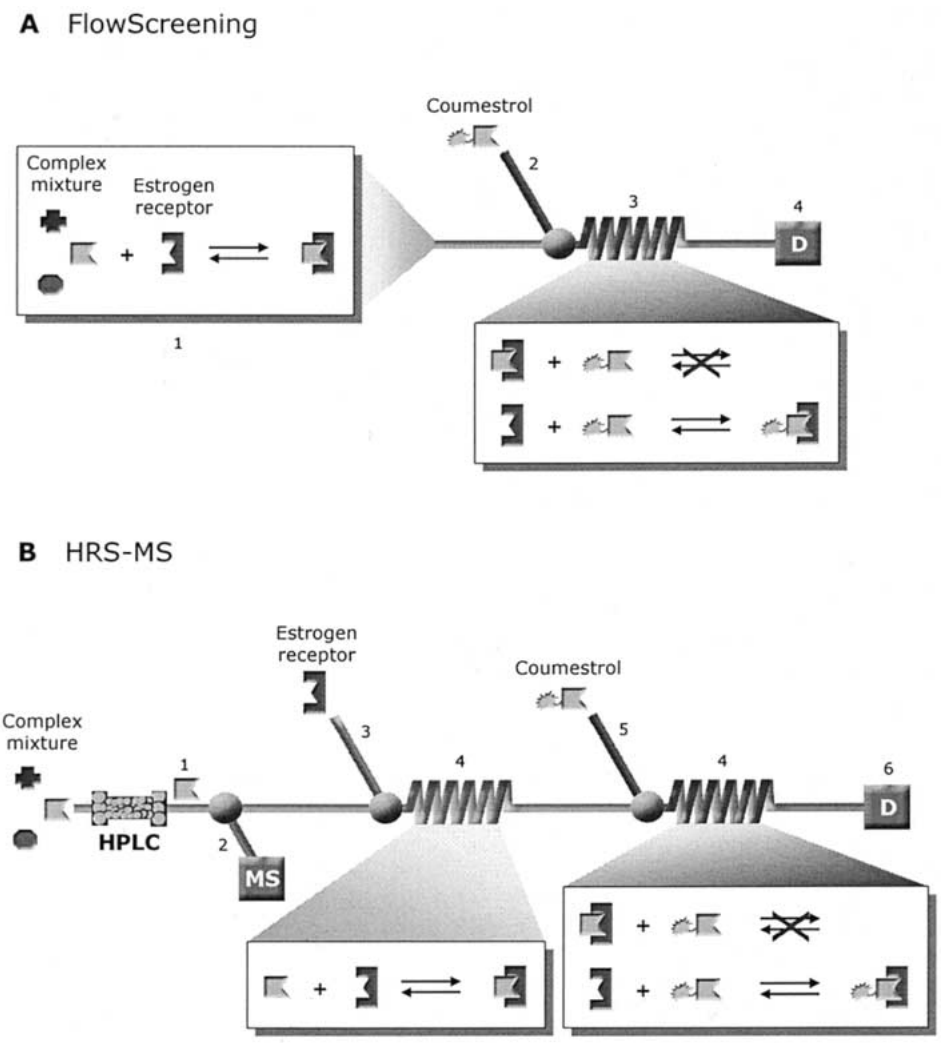

Figure 4. (A) Scheme of the FlowScreening configuration. 1. Autoinjector used for sample handling and selection. Injection into carrier solution; 2, Addition of coumestrol solution; 3, Reaction coil; 4, Fluorescence detector (D).
(B) Scheme of the HRS-MS configuration. 1. Effluent of the HPLC column; 2, Split to the mass spectrometer; 3, Addition of ER solution; 4, Reaction coil; 5, Addition of coumestrol solution; 6, Fluorescence detector (D).

Structure identification using LC-BCD-MS

Natural product extracts confirmed as bioactive in the flow injection assay were subsequently processed in the LC-BCD-MS mode. In order to allow the BCD and the MS to be monitored in parallel, the HPLC effluent was directed towards both detection systems, by introducing a flow splitter (1:20) after the analytical column (Figure 4B). Using LC-BCD-MS, bioactivity could be detected in 55% of all cases. Whenever bioactivity was found, 90% could be correlated to mass spectrometric information.

The structure elucidation of bioactive components in crude extracts is demonstrated by using the LC-BCD-MS data obtained from an extract spiked with 6 μM of α-zearalanol and an active extract of the Chinese medicinal herb *Kummerowia stipulacea* (Fabaceae). Figure 6 shows the chromatographic profiles of these extracts (left, α-zearalanol; right, *Kummerowia stipulacea*) as recorded by the LC-BCD-MS system. After injection of the extracts, the ER solution is added to the effluent stream of the HPLC as described previously. The enhanced coumestrol signal is stabilized within 2.5 min. The remaining window of 5.5 min. was used to detect active compounds

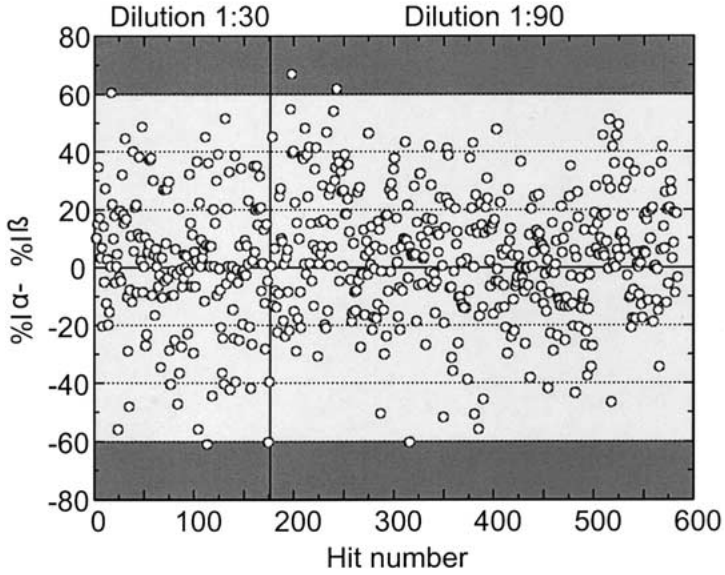

Figure 5. Scatter plot of differences between inhibition values for ERα and ERβ of all confirmed hits. Inhibition values used represent the percentage of maximum inhibition. The regions of ERα and ERβ selective hits, displayed on the positive and negative y-axis, are highlighted in gray

eluting from the HPLC-column. The two chromatograms clearly showed negative peaks at 5.50 min and 6.25 min. for α-zearalanol and *Kummerowia stipulacea*, respectively, which indicated the presence of bioactivity. Subsequently, the MS spectra, which corresponded to these particular retention times, were investigated for ions which exhibited a similar elution profile as the bioactive compound found in the BCD readout (Figure 7A). In this case, the MS-spectrum showed only a few relatively large peaks with intensities above the noise level, which allowed reconstructed ion current graphs to be prepared in order to assess retention times (Figure 7B). In this case, m/z = 321.4 and m/z = 327.4 for α-zearalanol and *Kummerowia stipulacea*, respectively, proved to be the only ions, which exhibited a retention time similar to the one observed by BCD. As no other ions could be found which indicated source decay of the parent molecule, a molecular mass of 322.4 g/mol (α-zearalanol) and a possible molecular mass of 328.4 g/mol (*Kummerowia stipulacea*) could be assigned to the bioactive compounds.

In addition, by operating the DECA mass spectrometer in the data dependent scan mode, an MS/MS fingerprint was obtained for m/z = 321.4 (α-zearalanol) and m/z = 327.4 (*Kummerowia stipulacea*) (Figure 7C). With the knowledge of the HPLC retention time, the molecular mass and the MS/MS fingerprint a database search can be performed to elucidate the structure of the bioactive compound. In the case of the extract spiked with α-zearalanol, the MS/MS fingerprint obtained was easily correlated with MS data reported in the literature (Jodlbauer et al., 2000).

Similarly, on-line biochemical detection coupled to mass spectrometry (LC-BCD-MS) was applied to rapidly profile estrogenic activity in a pomegranate *(Punica granatum*; Punicaceae) pericarp extract. Over the years, pomegranate has been demonstrated to contain the highest botanical concentration of the steroid estrone at 17 mg/kg dried seed (Heftmann et al., 1966). Campesterol and 17α-estradiol have been detected in seed oil (Kim et al., 2002), whereas coumestrol (Moneam et al., 1988), estriol and testosterone (Abd El Wahab et al., 1998) have been found in pomegranate seeds. Recently, fractions of the pomegranate, i.e., crude seed oil, crude fermented and unfermented juice and pericarp extract, were shown to exert anti-proliferative effects on human breast cancer cells *in vitro* (Kim et al., 2002). By applying LC-BCD-MS the crude pericarp extract was chromatographically separated, after which the presence of biologically active compounds, known or unknown, was detected by means of an on-line β-estrogen receptor bioassay. Using this approach in total three estrogenic compounds, i.e., luteolin, quercetin and kaempferol, were detected and identified by comparing the obtained molecular weights and negative ion APCI MS/MS spectra with the data of a phytoestrogen compound library.

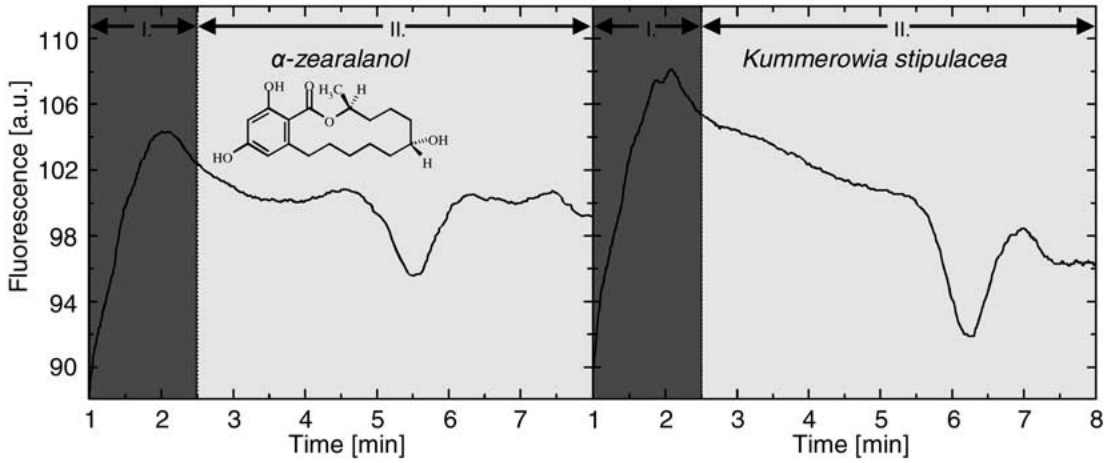

Figure 6. Bioactivity trace of the HRS analysis. (Left) Extract spiked with 6 μM α-zearalenone. (Right) Reconfirmed hit (extract of Chinese medicinal herb *Kummerowia stipulacea*). I: Equilibration of the enhanced coumestrol signal when ER is added to the flow system. II: Window used for detecting compounds eluting from the HPLC. Activity is detected as a reduction in coumestrol enhancement.

Confirmation of compound identity was obtained by analyzing reference compounds. The retention times of the bioactive compounds in the pericarp extract and those present in the reference solution were found to be almost identical (Figure 8A–B). Combined with the biochemical responses (Figure 8C) the identities were confirmed. Although well known in literature and widely distributed in nature, the presence of these phytoestrogenic compounds in pomegranate pericarp extract has not been reported previously.

MS based biochemical detection

Label-free detection

The use of mass spectrometry as the principal method for the detection of bioactive molecules, presents an alternative way of performing natural product screening. In the past fully automated LC-UV/MS systems have been reported, which enable detection of bioactive molecules based on affinity selection (Hsieh et al., 1996; Kaur et al., 1997; Lenz et al., 2000). Generally, the samples and target protein are incubated for a determined period of time after which affinity bound and unbound ligands are separated by means of size exclusion chromatography (SEC). The affinity complexes are trapped on reversed phase columns after which the affinity interaction is disrupted. Subsequently, the released ligands are eluted from the analytical column and analyzed by MS. As a result of the separation time required however, the use of SEC as a tool to separate affinity complexes from unbound molecules only allows high affinity molecules to be detected. In addition, analysis of complex matrices such as natural product extracts, which can contain macromolecules, could lead to erroneous results because of insufficient separation power provided by the SEC column. As a result, these approaches are generally more compatible with combinatorial compound libraries. Recently, a similar MS based affinity selection strategy based on restricted access columns (RA) was described (van Elswijk et al., 2001). Extracts, injected into a carrier phase, were allowed to interact for 2 minutes with the target protein, which was added to the carrier phase continuously. Subsequently, the affinity bound and unbound ligands were rapidly separated by a RA-column. The unbound ligands were trapped efficiently inside the small hydrophobic pores of the RA-material, whereas the bulky target proteins and affinity complexes passed the column almost unretained. Subsequently, the affinity complexes were dissociated on-line by adding an acidic solution to the mixture. By implementing a second RA-column the small ligands were trapped, whereas the target proteins and macromolecules passed the column. Trapped ligands were eluted and analyzed by MS. Using an anti-digoxigenin FAB/digoxin model system the principle of the method was demonstrated by the detection of digoxin in a spiked plant extract. Compared to similar approaches using size exclusion chromatography (SEC) the time required for separation of affinity bound and unbound ligands is reduced to several seconds. As a result of the reduction in

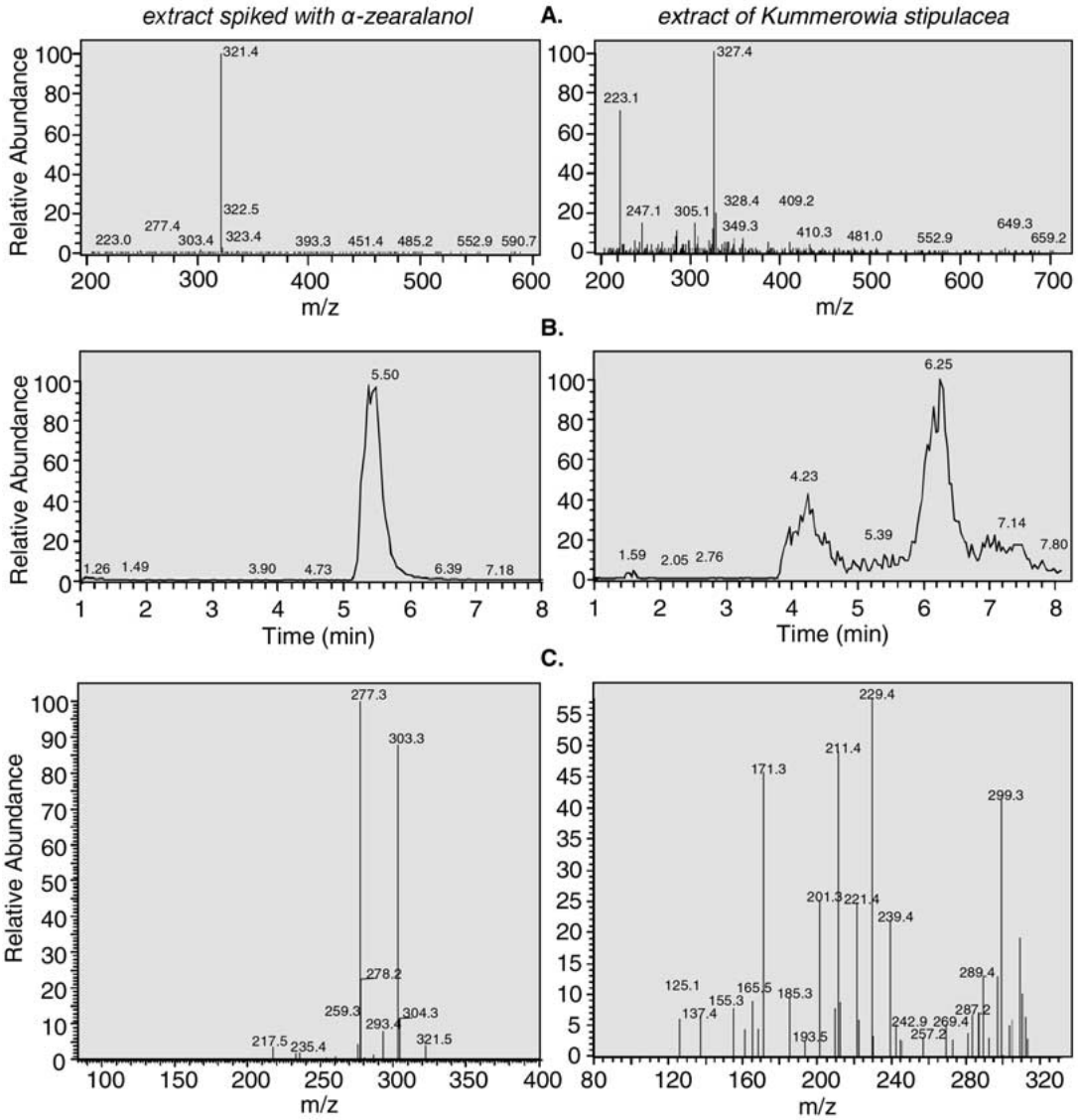

Figure 7. MS data recorded as a function of biological activity. (Left) Extract spiked with 6 μM α-zearalanol. (Right) Reconfirmed hit (extract of Chinese medicinal herb *Kummerowia stipulacea*). (A) The m/z pattern recorded at retention time of t = 5.50 min (α-zearalanol) and t = 6.25 min (*K. stipulacea*). (B) Reconstructed ion current of m/z = 321.4 (α-zearalanol) and m/z = 327.4 (K. stipulacea) for confirmning the retention time obtained in the bioactivity trace. (C) MS/MS spectra of the ions m/z=321.4 (α-zearalanol) and m/z = 327.4 (*K. stipulacea*) recorded in data-dependent mode.

affinity complex dissociation, bioassay sensitivity is enhanced. Moreover, by implementing RA-columns the interferences from macromolecular molecules are reduced significantly.

MS based monitoring of biospecific interactions

In addition to the label-free bioassay format, the ability of mass spectrometry to continuously monitor specific ions has been used to develop continuous-flow biochemical assays purely based on MS readout. Recently, Hogenboom et al. demonstrated the possibility of monitoring biospecific interactions with soluble target proteins using electrospray mass spectrometry (Hogenboom et al., 2001). The biochemical detection system was based on a solution-phase, homogeneous assay. In a first step, biotinylated compounds were injected into a continuous-flow reaction system and allowed to react with the affinity pro-

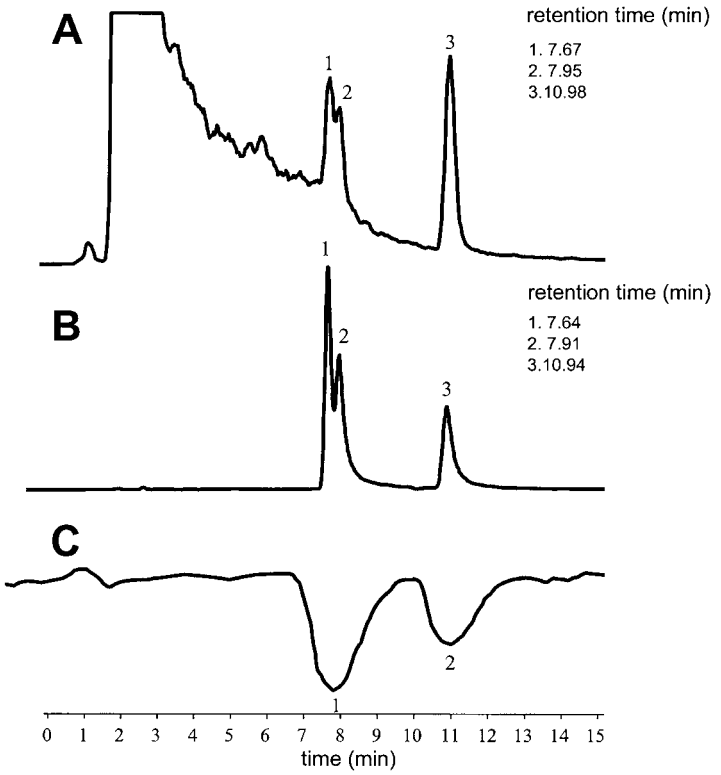

Figure 8. Identity confirmation of phytoestrogenic compounds found in pomegranate extract. 1. luteolin, 2. quercetin, 3. kaempferol. A: RIC of acid hydrolyzed extract – m/z 285.5 and 301.5. B: RIC of reference solution containing luteolin (260 μM), quercetin (280 μM) and kaempferol (240 μM). C: Bioactivity profile of reference solution.

tein, streptavidin. Subsequently, a reporter ligand, fluorescein-biotin, was added to saturate the remaining free binding sites of the affinity protein. The concentration of unbound reporter ligand was measured using electrospray MS in the selected-ion monitoring mode. The presence of active compounds in the sample resulted in an increase of the concentration of unbound reporter ligands. The feasibility of a homogeneous MS-based biochemical assay was demonstrated using the described streptavidin/biotin system as well as an anti-digoxigenin/digoxin model assay. For the biospecific interaction studies, detection limits of 10–100 nmol/l were obtained. An important advantage over the fluorescence-based approaches is that the synthesis and purification of labels is bypassed. In cases where these fluorescent labels are not available, the MS based bioassay represents an alternative way of screening for bioactive compounds. Compared to the fluorescence-based approaches, monitoring biospecific interactions by MS requires careful selection of buffer conditions. Typically used buffer solutions in biochemical detection contain phosphate, sodium chloride and detergents, which profoundly influence the ionization efficiency of the MS detector. As a consequence, more appropriate buffer solutions, like acetate and carbonate, are used in order to enhance assay sensitivity.

Conclusion

The methods described in this paper demonstrate the advantages of technologies which provide both biological and relevant chemical data in parallel during a single sample analysis. Not only is there a significant decrease in resources required, but a profound reduction of the time needed for compound description in complex mixtures such as natural product extracts is achieved as well. The wide range of bioassay formats available enables screening of a multitude of diverse protein targets. With the advent of a vast number of new molecular targets and the increasing demand for new leads in HTS, incorporation of natural products, as an additional and major source of novel

drug-like molecules, and efficient screening technologies, like LC-BCD-MS, could prove to be of great pharmaceutical and commercial value.

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