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Current Opinion in Biotechnology

Microscale methodology for structure elucidation of natural products

Tadeusz F Molinski

Advances in microscale spectroscopic techniques, particularly microcryoprobe NMR, allow discovery and structure elucidation of new molecules down to only a few nanomole. Newer methods for utilizing circular dichroism (CD) have pushed the limits of detection to picomole levels. NMR and CD methods are complementary to the task of elucidation of complete stereostructures of complex natural products. Together, integrated microprobe NMR spectroscopy, microscale degradation and synthesis, are synergistic tools for the discovery of bioactive natural products and have opened new realms for discovery among extreme sources including compounds from uncultured microbes, rare invertebrates and environmental samples.

Address
Department of Chemistry and Biochemistry and Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, 9500 Gilman Drive MC0358, CA 92093, United States

Corresponding author: Molinski, Tadeusz F (tmolinski@ucsd.edu)

Current Opinion in Biotechnology 2010, **21**:819–826

This review comes from a themed issue on
Pharmaceutical biotechnology
Edited by William Fenical and Russell Hill

Available online 27th September 2010

0958-1669/$ – see front matter

DOI 10.1016/j.copbio.2010.09.003

Introduction

Natural products, the small organic molecules produced by many microbes, plants and invertebrates, are the source of some 50% of modern drugs [1•]. New opportunities present themselves for natural product discovery that take advantage of multi-pronged approaches to drug discovery including high-throughput screening, gene sequencing, metabolic engineering and synthetic biology [2••,3••]. Modern molecular genetics now makes possible the sequencing of entire genomes of organisms: the blueprints for all things living and, ostensibly the biosynthetic masterplan of natural product structures. State-of-the-art of sequencing of natural product biosynthetic genes and predictive 'gene gazing' [4,5••] will no doubt eventually enable structure elucidation from sequence and biosynthetic context, alone. So far, however, the latter approach has predicted the structures of only a few natural products and falls short in applicability, partly due to the complexity of the multi-enzymatic pathways of secondary metabolism, which differ widely from one class of natural product to another, and a lack of understanding of detailed information of the respective enzymes, their regulation and their capacities to channel substrates into products. Consequently, even in the modern age, the structures of natural products are largely still determined by conventional protocols: integrated, systematic application of spectroscopic methods (mass spectrometry; MS, electronic spectroscopy; UV–vis, infrared spectroscopy; IR, nuclear magnetic resonance; NMR, specific optical rotation; $[\alpha]_D$, and circular dichroism; CD and X-ray crystallography.

X-ray crystallography is the ultimate tool for molecular structure determination. Regrettably, the majority of natural products do not produce suitable X-ray quality crystals, and integrated spectroscopic methods are still the most practical means to structure elucidation. The fundamental weak link in this chain is NMR, the most powerful yet the least sensitive method. Nevertheless, impressive accomplishments in structure determination of extremely small sample amounts of natural products have been realized, largely by 'pushing the limits' of the prevailing NMR technology of the time. For example, the structure elucidation of ciguatoxin — a complex polyether toxin responsible for mass food poisonings from ingestion of toxic fish — was completed in 1990 with only 0.3 mg of sample, purified from over two tons of fish viscera, using 600 MHz NMR and room temperature 5 mm probes [6]. (For an informative review of this and other representative examples of sub-milligram scale natural product structure determinations, see Murata and coauthors [7].)

Time-averaging of signal improves the detection limits of NMR, but pragmatic concerns of instrument duty cycles and time management do not allow indefinite signal acquisition. Of greater concern is dynamic range — the ability to detect sample signal over background signal (solvent, impurities, *etc.*). Both concerns place bounds on the practical limits of structure determination of natural products of limited sample size. Until a few years ago, the practical working limit was around a micromole (10^{-6} mole, or ~1 mg for a compound of molecular mass of 1000), but recent revolutionary changes in NMR instrumentation have pushed this limit down to only a few nanomole (10^{-9} mole). The key developments in commercial NMR instrumentation that have made this possible include advent of smaller volume probes (1 mm capillary probes and 1–1.7 mm microtube probes)

coupled with cryogenically cooled preamplifier electronics that reduce electronic noise (*N*) and increase signal (*S*), respectively, with an attendant increase of 10–20-fold in signal-to-noise ratio (*S*/*N*). Several recent reviews showcase the advantages of cryomicroprobe and capillary probe NMR in natural product studies [8•,9,10]. Capillary NMR flow probes are particular well-suited to integration with hyphenated LC–MS–NMR platforms for high-throughput screening [11], or rapid dereplication protocols [12], but dynamic range is still a limitation, at least for the NMR component. As an added advantage, capillary and cryomicroprobe NMR spectroscopy can reveal previously hidden chemical diversity of natural products within extracts from a single organism by powerful coupling of component analysis by high-dynamic range HPLC with NMR interrogation of vanishingly small peaks.

While applications of multidimensional NMR methods routinely reveal the molecular constitution formula of natural products, no general solution exists to the problem of assignment of relative and absolute configuration; absolute stereostructures are elucidated on a case-by-case basis. Circular dichroism (CD), a well-known biophysical technique for protein secondary structure determination, also boasts a long history in chiroptical analysis of organic molecules. The advantages of CD over optical rotation measurements for stereostructure assignment include high sensitivity (low sample requirement) and linearity with concentration (CD obeys the Beer–Lambert law). In recent years, the refined numerical methods for calculation of CD spectra by time-dependent density functional theory (td-DFT) and low-cost computing power make possible configurational assignments of natural products by matching the measured spectra [13–16].

Lastly, after application of methods that secure the complete structure of a nanomole of natural product, what can one do with it? Preliminary biological evaluation may require samples of several milligrams while preclinical trials demand grams to kilograms. Here, the power of modern gram-scale multi-step organic synthesis dovetails nicely with nanomole-scale discovery of natural products. For example, the first finding of discodermolide, a promising anticancer drug obtained from a rare deep-water sponge, *Discodermia dissoluta*, resulted in only a 7 mg yield. Recollections of the sponge with deep-water submersibles were not sustainable, however, the compound was secured in 60 g amounts by total synthesis [17••].

HPLC and nanomole-scale NMR may also reveal unexpected chemodiversity and biodiversity among familiar organisms. For example, the sponge-eating tropical dorid nudibranch, *Hexabranchus sanguineus* — a shell-less sea-slug also known as the 'Spanish dancer' — sequesters a large amount of cytotoxic 'trisoxazole' macrolides (over 160 mg per slug! [18]), including kabiramide C [19]. Narrowing the focus to the very minor components in the extract leads to an unexpected finding: new modified peptides sanguinamides A and B, in sub-milligram yields [20]. Because *H. sanguineus* prefers a diet of trisoxazole-containing sponges, the latter suggests a more complex secondary metabolite input, perhaps other dietary sponges that contain the new peptides.

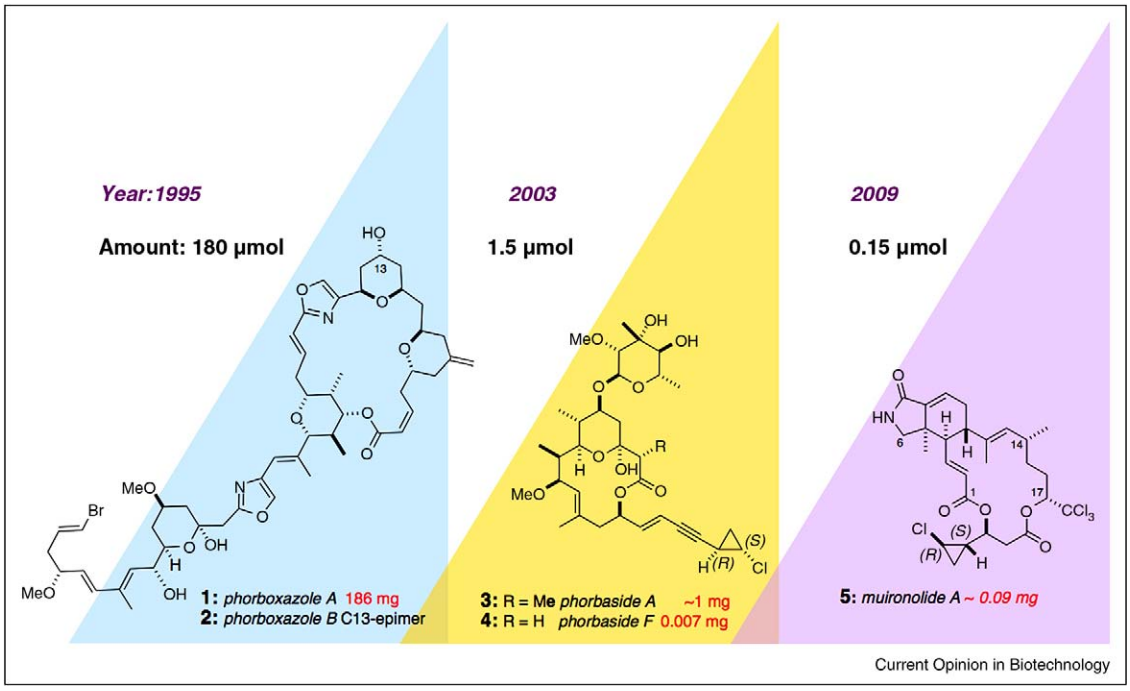
Nano-mole scale natural product chemistry — microcryoprobe NMR

Natural product chemistry, for the purpose of finding therapeutic leads, is a screening strategy based upon numbers. High throughput screening platforms that assay large panels of extracts, fractions or pure compounds against clinically relevant screens that target disease is time efficient strategy. Yet imperfections in screening protocols may only favor identification of the 'low hanging fruit' and lead to missed opportunities. These practical realities are well appreciated by practitioners of the art, but missed drug 'hits' that fall below the thresholds of detection in screening campaigns may be repositories of those very interesting new chemical entities (NCEs) — drug-like molecules with unexpected, new chemical structures.

For the purposes of this review, the power of nanomole-scale natural product discovery can be nicely illustrated by the history of discovery compounds from a *single sample* of a new species of marine sponge, *Phorbas* sp. which has given rise to a remarkable number of unprecedented compounds, including phorboxazoles, phorbasides, hemi-phorboxazole and muironolide A. The structures of phorboxazoles A (**1**) and B (**2**) (Figure 1), exquisitely potent cytostatic agents with sub-nanomolar activity against a range of tumor cell lines, were established from NMR analysis using a 500 MHz NMR spectrometer equipped with a conventional inverse-detection 'room temperature' probe. Phorboxazoles were relatively plentiful — about 180 mg (~0.2 mmole) from about 200 g of sponge. The absolute stereostructures of **1** and **2** were revealed from data obtained from a combination of 1D and 2D NMR experiments [21], synthesis of model analogs, Mosher's ester derivatives [22], and — for C-43 — degradation of the side-chain to (*R*)-tri-*O*-methyl malate, followed chiral GC analysis [23]. Later, with access to improved instrumentation (600 MHz, 5 mm cryoprobe NMR), the unrelated phorbasides A (**3**), B [24], C–E [25], were uncovered (0.1–2.7 mg) from minor chromatography side-fractions. Chiroptical analysis of **3** by quantitative CD and comparison with prepared model compounds of known configuration, were used to assign the configurations of the remote stereocenters C-19, C-20 of the 2-chlorocyclopropane unit [24]. This assignment was verified by the recent synthesis of phorbaside A (**3**) by Paterson and Paquet [26].

In mid-2007, the first commercial 1.7 mm 600 MHz cryomicroprobe became available, and the *most minute* fractions

Figure 1



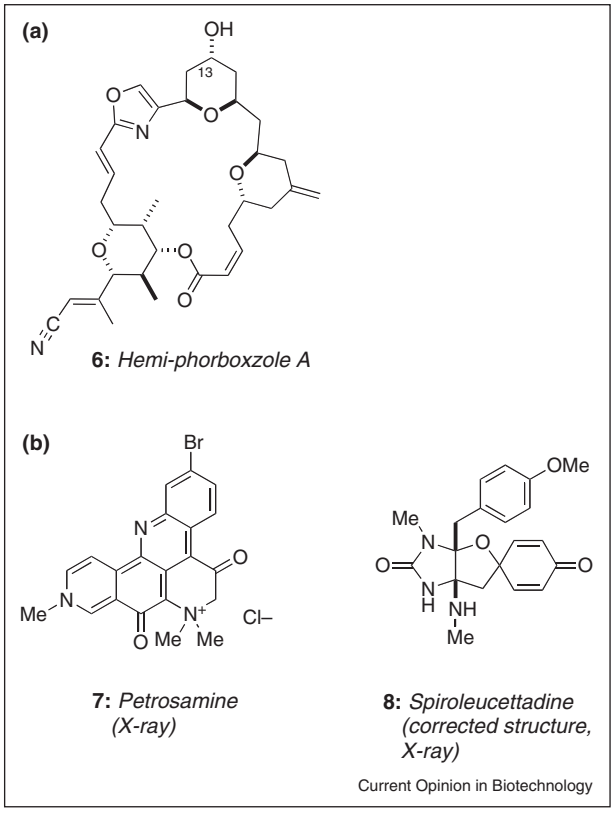
'Three generations' of macrolide natural products from a single sample of the marine sponge, *Phorbas* sp.

from the *Phorbas* extract could be examined. Phorbasides F (**4**) [27], G–I [28] (7–16 µg) were isolated along with two unexpected molecules: muironolide A (**5**) [29] (90 µg) and hemi-phorboxazole A (**6**, 16.5 µg, Figure 2). Muironolide A is an unprecedented macro-triolide containing an 1*H*-isoindolin-1-one ring system, a trichloromethyl carbinol ester and a 2-chlorocyclopropane, was shown to be *opposite* in configuration to that of the phorbasides A and B [24]. Hemi-phorboxazole A (**6**) [30], the third member of the phorboxazole family, was fully characterized from a *total* sample of only 16.5 µg. Because **6** is approximately half the molecular mass of **1**, it is likely to arise as an oxidative degradation product of phorboxazole A or biosynthesized as a truncated polyketide, and terminated in a similar manner to that of borrelidin, an α,β-unsaturated nitrile produced by several species of *Streptomyces* [31,32]. With the complete stereostructure of the natural product in hand, a short synthesis of **6** [33] subsequently provided milligrams of material, sufficient for biological evaluation. In the course of these investigations, a practical NMR method was refined for 'in tube' quantitation of natural product samples [27], which proved useful for further measurements of molar spectroscopic quantities (e.g. UV–vis molar absorbtivity, ε, or molar circular dichroism, Δε) after quantitative sample recovery.

It is worth reminding that the provenance of the foregoing compounds was a single specimen of *Phorbas* sp.; notable for its rarity and lack of successful recollection [34•].

Current trends in NMR development indicate that future gains in *S/N* will be expected, particularly for insensitive spins such as ^{15}N and ^{13}C. One promising technique, although first described decades earlier, is dynamic nuclear hyperpolarized NMR (DNP) [35,36] that exploits the advantage of the large difference between ground state and excited states of unpaired electron spins (radicals), and transference of the population difference to NMR active nuclei. Polarization of spin nuclei is achieved in two stages: pumping rf energy into the electron spin energy levels of a co-mixture of sample and stable organic radical in a cryogenically frozen matrix using microwave radiation (GHz). Favorable polarization transfer populates the ground state (α) of the nuclear spins and depopulates the excited state (β); a subsequent rf pulse at the NMR frequency (MHz) results in greatly improved NMR signal intensity, particularly for inherently insensitive spins (up to 10^4-fold improvement in *S/N* for ^{13}C). Disadvantages of DNP NMR include the expense of hardware (a separate microwave transmitter and cryomagnet is required for *ex situ* polarization, and pump lines for sample thawing and rapid transfer into a separate NMR cryomagnet) and the ephemeral nature of the hyperpolarized nuclear spin states. Only a limited acquisition time window (~1 s) is available to measure the NMR of 'pumped' nuclei before the spins return to their equilibrium Boltzmann population. Nevertheless, new pulse sequences for ultra-fast 2D NMR experiments [37] partly overcome the limitation, and favor adaptation

Figure 2



(a) Hemi-phorboxazole A (**6**). **(b)** Structures of 'proton-poor' alkaloids (H/C < 2) petrosamine (**7**) [39] and spiroleucettadine (**8**) [40].

of DNP to 2D ^{1}H–^{13}C NMR (HSQC, HMBC) of natural product characterization [38]. One desirable application of DNP is the enhancement of long-range heteronuclear ^{1}H–^{13}C HMBC correlations ($^{4}J_{CH}$, $^{5}J_{CH}$) where limited sample, lack of *S*/*N* and an unfavorable ratio of H/C in the empirical formula militate against structure elucidation by 2D NMR spectra. For example, both the polycyclic alkaloids, petrosamine (**7**, Figure 2), from *Petrosia* sp. [39] with formula $C_{21}H_{17}BrClN_3O_2$ (H/C = 0.8) and spiroleucettadine (**8**), from *Leucetta* sp., ($C_{20}H_{23}N_3O_4$, H/C = 1.2) are, 'meager in H atoms'. Both compounds violate the so-called 'Crews Rule'; a required ratio H/C > 2 that allows a sufficient number of 2D long-range heteronuclear correlations to complete structure elucidation [40]. The correct structures of both compounds, in fact, were solved by X-ray crystallography.

Chiroptical techniques: circular dichroism and absolute configuration

Optical rotation and circular dichroism are traditional methods for the characterization of chiral optically active natural products. Advance optical rotation, on the basis of laser interferometry, can theoretically, allow detection of picograms of material in low-volume samples (~12–40 nL) and low limits of detection of rotation (4×10^{-4} degrees) [41]. Detection of optical rotation, α, by laser optical methods and flow techniques is adaptable to HPLC for on-the-fly characterization of chiral molecules [42,43]. Circular dichroism (CD) is observed when circularly polarized light (CPL) passes through a solution of a chiral, optically active compound whose molecular structure contains a chromophore. The advantages of CD over polarimetry include high-sensitivity, and less interference from solvent effects and birefringence artifacts. CD may be thought of as differential molar absorptivity, $\Delta\varepsilon$, of chromophores absorbing left and right CPL according to Eq. (1); the resultant light is elliptically polarized light and simple linear relationship exists between $\Delta\varepsilon$ and molar ellipticity, $[\theta]$. CD uses dilute solutions that obey the Beer–Lamber law and is better suited than specific rotation, $[\alpha]$, for quantitation. The so-called asymmetry factor, *g*, (sometimes called 'anisotropy factor', Eq. (2)) is a measure of the magnitude of $\Delta\varepsilon$ as a ratio of ε and a convenient measure of the 'strength' of dichroism in chiral materials.

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R \quad 3300\Delta\varepsilon = [\theta] \tag{1}$$

$$g = \frac{|\Delta\varepsilon|}{\varepsilon} \tag{2}$$

Traditionally, CD has been used in organic spectroscopy for assignment of absolute configuration by the observation of Cotton effects (CEs), based either on *empirical* 'sector rules' or on the sign of split-CEs from *non-empirical* exciton coupled CD (ECCD) [44]. Two areas of application of CD to natural product analysis are of interest: analytical quantitation of enantiomer composition and structure elucidation by interpretation of CEs. HPLC detection by LC–MS–CD, using optical rotation detectors and CD detectors has been used for chiroptical analysis of dichroic compounds [45]. Because $\Delta\varepsilon$ is relatively small compared to ε, the sensitivity of CD-HPLC is dependent upon *g* (*g* values are commonly in the range 0–10^{-3}). Consequently, the sensitivity of the CD-detected HPLC is lower than traditional fluorescence or UV-detected HPLC, and better detection limits will be realized for natural products with higher *g* values.

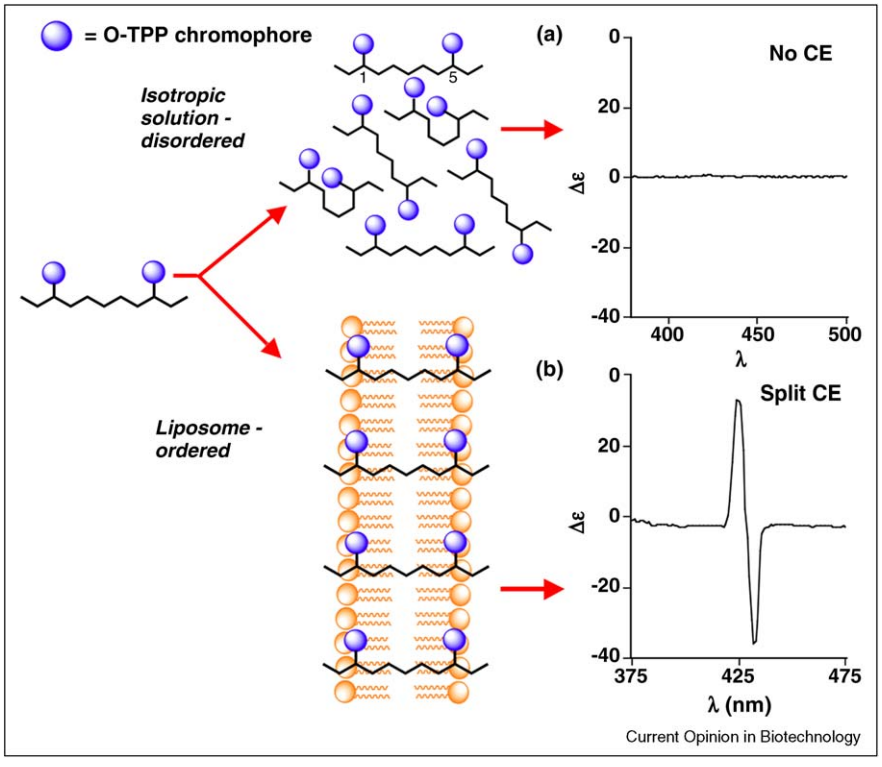
Integration of CD-detected HPLC traces, using columns with chiral stationary phases (e.g. Chiracel®, Chirex® or Pirkle-type columns), gives signed peak intensities of resolved enantiomers whose integrals identify both optical purity (enantiomeric excess, %ee) and the sign of $\Delta\varepsilon$ for the major enantiomer [46]. Conversely, CD-detected HPLC with *achiral* columns can be used for the determination %ee of known molecules with well-characterized *g* values by simultaneous measurement of CD and UV spectra, rationing of the ε and $\Delta\varepsilon$ signals and comparison with *g values of* the pure compound [47–49]. HPLC with CD detection has been used in quantifying

enantiomeric ratios for a variety of synthetic molecules, and has potential applications to natural product analysis, particularly with compounds that exist as non-racemic mixtures of enantiomers. This is particularly useful under variable methods of HPLC analysis of enantiomers or closely related analogs where, although the elution order of analytes may change, the sign of the CD signal of enantiomers remains the same. Because of dynamic range and sensitivity issues, reliable quantitation of enantiomers by HPLC CD on non-chiral stationary phases is limited to those analytes with optical purities <99%ee [49].

Peaks in CD spectra, referred to as Cotton effects (CEs), are observed only in chiral molecules containing chromophores, but the CD spectra of acyclic chiral molecules which are conformationally mobile with a relatively large number of degrees of freedom, or where the chromophore is remote from the elements of asymmetry, may also be weak or zero. The latter limitation has been overcome recently with the use of *liposomal circular dichroism* (L-CD) in which the sample to be measured is formulated in uniform, unilamellar liposomes. Under these conditions, acyclic molecules become ordered within the liposomal lipid bilayer and CEs are greatly amplified (Figure 3). The latter has been exploited for enhanced ECCD determination of both *relative* and *absolute* configuration of tetraphenylporphyrincarboxylate diesters (TPP esters) of 1,5-diols, 1,7-diols and 1,9-diols [50,51].

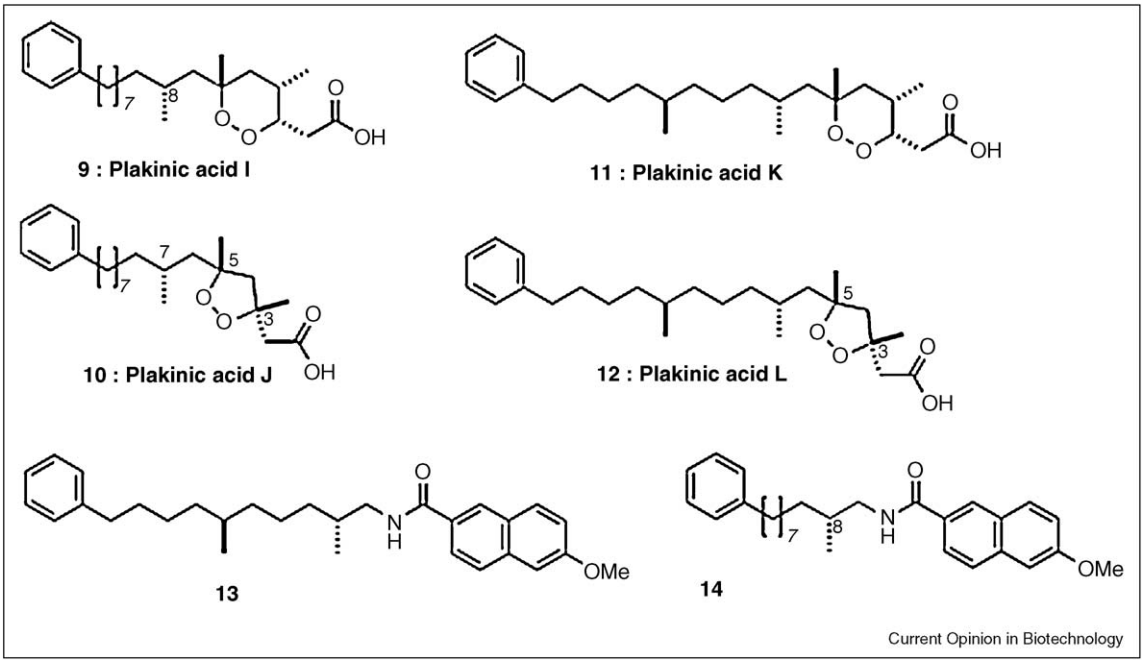
Molecules lacking chromophores in their UV–visible spectra can be interrogated by CD after derivatization. Within the past year, L-CD has been applied to molecules containing only a *single* chromophore located remotely from the asymmetric centers. For example, the configuration of remote single-methyl-branched and double-methyl-branched stereocenters in acyclic polyketides has been assigned by L-CD. Plakinic acids I (**9**), J (**10**) [52], K (**11**) and L (**12**) [53] (Figure 4) are potent antifungal agents isolated from a two-sponge association of *Plakortis halichondroides–Xestospongia deweerdtae*. The configuration of stereocenters within the 1,2-dioxane ring were solved by conventional methods, however, the remote methyl-branched stereocenters in the side-chain were more problematic. Reductive free-radical mediated cleavage of **9**–**12** with iron (II) chloride, under oxygen-free conditions, liberated a primary chloroalkane which was converted in three steps to naphthamide **13** or **14**. The CD of **13** in MeOH showed only baseline spectrum (cf. Figure 3a), however, when **13** was formulated in liposomes prepared from distereoyl-*sn*-glycerophosphatidyl choline (DSPC), the CD spectra of **13** and **14**,

Figure 3



Liposomal CD (L-CD). Exciton coupling amplified by ordering or lipid chains in liposomal bilayer. **(a)** CD of a long-chain 1,5-diol tetraphenylporphyrincarboxylate (TPP) diester (MeOH). **(b)** L-CD spectrum (DPSC liposomes). See Refs. [50,51].

Figure 4



Plakinic acids I–L (**9**–**12**) from a *Plakortis–Xestospongia* sponge association, and derived naphthamides **13** and **14** for liposomal CD (L-CD).

revealed strong exciton coupled CEs that could be matched to those of a synthetic model compound [52,53]. Moreover, diastereomeric CE differences allowed discrimination of all four possible stereoisomers of **14** and assignment of complete configurations of **9**–**12**.

The success of L-CD relies on ordering of long-chains within the membrane bilayer as demonstrated by temperature-dependent CE. Above the gel transition temperature of DSPC bilayers ($T_C = 54.5°C$), the CE dramatically diminishes — almost to zero — but recovers upon annealing back to room temperature [53].

L-CD is ideally suited for difficult assignments in long-chain acyclic natural product molecules containing remotely spaced stereocenters. Although L-CD is a nascent development, the obvious advantages in chiroptical analysis of long-chain acyclic polyketides make it an attractive subject for future exploration and refinement.

What is the future of microscale natural product structure elucidation? As an illustration, consider the ultimate limit of detection — a single molecule. Conventional fluorescence-detected microscopy can detect *the presence* of single molecules, if adorned with appropriate fluorophores. Imaging techniques such as atomic force microscopy (AFM) and scanning tunneling microscopy (STM) now routinely reveal atomic-level features and patterns on the surfaces of ordered solids, and even single molecules deposited upon smooth surfaces. A dramatic improvement was realized in 2009 with AFM based on 'Pauli-exclusion' forces using AFM probes with atomic precision, fashioned by attachment of a single molecule (e.g. CO) to the probe tip [54]. The molecular features of single molecules of pentacene were revealed in unprecedented detail, down to visualization of individual C–H bonds and contrast levels ordered by π-orbital density. How soon will we be able to 'see' a single natural product molecule by AFM imaging — visualization and identification of atoms from orbital density, bond connectivity and bond order from interatomic distances, even stereochemistry! — and simply transcribe the details to a written structural formula, much as an artist sketches a model?

Conclusions

Applications of modern innovations in organic spectroscopy, particularly NMR and CD, have expanded the reach of the natural product chemist and revealed the molecular structures of compounds available only in vanishingly small amounts. The effect of this range expansion — from sub-millimole to nanomole — broadens the scope discovery of new natural products to rare organisms, particularly uncultivated microbes and environmental samples, and reveals chemodiversity within single specimens. Finally, innovations in AFM that allow visualization of single molecules, with atomic-level precision, may soon realize an application to natural products — the provocative concept of 'seeing' a single molecule and solving the molecular structure of an unknown using no more than visual inspection of atoms and bonds.

Acknowledgements

I am indebted to the dedicated graduate students and postdoctoral fellows in my laboratory, whose meticulous and tireless efforts made possible many of the accomplishments described in this review. The author is grateful to the National Institutes of Health (CA122256; AI039987) for funding.

Note added in proof

A systematic investigation of the experimental limits of small-sample heteronuclear 2D NMR with a commerical 1.7 mm microcryprobe has been appeared [55].

The first structural investigation of a natural product (cephanolide) A by enhanced AFM microscropy has appeared [56].

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- • of special interest
- •• of outstanding interest

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