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Isolation of Microbial Natural Products

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Summary

Microbial fermentations conducted with the express purpose to generate organic molecules are invariably carried out in a liquid medium, and the organic components produced can be distributed between both the solid and liquid phases. A major challenge facing the practitioner attempting to isolate a microbial natural product, where the organic component is extracellular and has been exuded into the medium, is the separation of the desired organic components from the aqueous broth that constitutes the majority of the mass. This chapter places natural products in a medicinal context from a historical perspective, demonstrating that biological activity in natural compounds has a long history. It subsequently goes on to summarize, using appropriate examples, two methods invaluable in the isolation of microbial natural products. The techniques considered are liquid–liquid extraction concentrating on countercurrent methods of separation and liquid–solid extraction focusing on the use of polymeric adsorbents in separation.

Key Words: Natural products; fermentation; isolation; microbial; countercurrent chromatography; countercurrent distribution; liquid ion-exchange extraction; polymeric adsorbents; diaion.

1. Natural Products in Context: From Asclepius to Ehrlich

The isolation of natural products as marketable commodities is not a new phenomenon and has a history dating back many millennia. The fermentation of fruit and grain to produce the catabolic product we know

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as ethanol has its origins in prehistory, while more recently it is known that many cultural groups, including the Celts, the Greeks, and the Egyptians, kept records of production and consumption of alcoholic beverages. Until 1856, when William Perkins established what was to become the synthetic dye industry, the production of dyes was predominantly based on natural products. In addition to the multitude of plant-based colorings, a dyestuff called Tyrian purple, although not natural itself but rather a degradation product based on the naturally occurring tyrindoxyl sulfate, formed a major industry over 3000 yrs ago. Perhaps the most remarkable natural product traded in its pure form is sugar. In 327 BCE, Alexander the Great introduced sugar-cane and de facto sucrose into the Mediterranean. Sucrose is one of the most common and well-documented natural products known today with over 130 billion kgs produced in over 100 countries around the world each year.

Today the driving force behind natural product chemistry as a discipline is still largely predicated on the attainment of an economically viable product, often a therapeutic. As a consequence, much of the recent scientific research directed toward the isolation and structural elucidation of natural products has revolved around the quest for a cure. Until modern times, every civilization throughout history had relied upon the biological properties of natural products to stave of disease and prevent illness. This approach changed with the beginning of the 20th century, when Ehrlich and Hata ushered in a new paradigm for drug discovery, that of chemotherapy, involving an ordered search of a library of pure chemicals for a predetermined activity. Through this structured technique, they developed the synthetic chemical arsphenamine, an arsenic-based compound traded as Salvarsan, which was capable of destroying the syphilis pathogen, *Treponema pallidum*. Today such is the perceived success of this modern discovery paradigm that natural medicines have been largely relegated, at least in most of Western society, to that of complementary status, and to a large extent there is a perception that medicines available presently are synthetic chemicals.

A vast proportion of the population consider drug discovery to be a recent concept that evolved from modern science in the 20th century, whereas in reality it dates back many centuries and has its origins in nature. From a Westcentric perspective, the foundations of science and ergo medicine are considered to have been laid during the "Greek Golden Age," which reached its peak around the 5th century BCE. Indeed, the very word *medicine* along with *hygiene* and *panacea* are derived from the

Fig. 1. A representation of the "staff of Asclepius."

names of three of the daughters of Asclepius, Meditrina (Iaso), Hygieia, and Panacea, respectively. Asclepius himself was a physician and was considered to be the son of Apollo. He was worshipped as the Greek god of healing. The medical symbol frequently used today depicting a single snake coiled around a pole is based on the staff of Asclepius (**Fig. 1**).*

During the age of Greek domination of the sciences, a new mode of thought concerning nature was developed, and this became the foundation of modern observational science resulting in a more rational and logical approach to medicine and consequently drug discovery. Empedocles (504–443 BCE) extended the philosophy of Thales (639–544 BCE) and introduced the concept that four fundamental elements—air, earth, fire, and water—were the basis of all things *(1)*. It was into this society where reasoning was centered on the four "humors" as postulated by Empedocles that Hippocrates (460–370 BCE) was born. Considered the Father of Medicine, his methods of observation, scientific assessment, and therapeutic practice were the first of their kind, and he produced a range of medical texts, including those on treatments using many naturally derived drugs,

* There are two frequently used symbols for medicine. One is the staff of Asclepius (**Fig. 1**), which depicts a single snake coiled around a staff, while the second is the caduceus of Hermes and involves two serpents coiled around a staff capped by a pair of wings. Given that we associate the symbol with medicine, it would seem more appropriate that the staff of Asclepius be used as the symbol. On the other hand, the caduceus of Hermes (Hermes is the messenger of the gods and conductor of the dead) became associated with healing around the 7th century CE and was linked to alchemy, from which chemistry and chemotherapy evolved. Alchemists at the time were frequently referred to as Hermetists.

that were unsurpassed in their field for many centuries *(1)*. Theophrastus (372–287 BCE), who upon the death of Aristotle (322 BCE) inherited both his library and most importantly the botanical garden, is credited with creating the first-written herbal, an extensive and precise description of 455 plants and their medicinal properties. Another significant work, which built upon those of his forebears, was *De Materia Medica* written by Pedanius Dioscorides, who lived in the first century of the current era. The work was a study on the preparation, properties, and testing of drugs, and included over 1000 drugs of natural origin. Dioscorides' *De Materia Medica* was republished many times up until the 16th century and was the basis for the transmission of the recorded knowledge of medicine and drugs throughout the Dark Ages of Europe *(1)*. Arguably, the final great European figure in the drug discovery field before the Renaissance was Galenus (131–201 CE), better known as Galen. The first leading figure in experimental physiology, Galen produced more medical texts than any other ancient medical author. He created "galenicals," mixtures of herbs that were used as remedies until the advent of drugs that consisted of a single agent. With his death in 201 CE, scientific medicine declined in Europe for more than a thousand years. It was during this time that the Arab culture of northern Africa, Moorish Spain, and the Middle East established comprehensive libraries and founded hospitals and schools of learning, thereby advancing the scientific knowledge that was to be adopted in Europe with the Renaissance. One of the great Moorish scholars, al-Baitar (1188–1248 CE), built on the knowledge recorded by Dioscorides and Galen in the field of medicinal plants and assembled an extensive textbook on pharmaceuticals documenting over 1400 drugs *(2)*.

The science of natural products as distinct chemical entities with known molecular architectures is a relatively new field having commenced with the birth of organic chemistry as a discipline. The biggest breakthrough in isolation and identification of natural products in the last three decades has been a combination of the widespread availability of high-performance liquid chromatography (HPLC) instrumentation (*see* Chaps. 8 and 9), a topic recently reviewed in the first edition of this book by Stead *(3)*, and the advances in analytical instrumentation. Recent progress in nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) has revolutionized natural product chemistry, improving dereplication methods (*see* Chap. 12) and allowing novel compounds to be targeted and identified in a time frame of days on as little as a single milligram of compound. The isolation of microbial natural products involves a multitude of

techniques, and as such a single chapter can never be hoped to accurately reflect the breadth of techniques involved. However, in many ways, the isolation of microbial natural products does not differ significantly from the isolation of secondary metabolites from plants or animals. This chapter looks at two aspects, countercurrent methods and polymeric adsorbents, used extensively in microbial natural product chemistry.

2. The Isolation of Penicillin: The Beginning of Counter Current Chromatography

In any review concerning the isolation of microbial natural products, mentioning penicillin is not unexpected. While penicillin was not the first compound to be isolated from a micro-organism, indeed in the context of drug discovery that honor would probably belong to the isolation of mycophenolic acid obtained from *Penicillium brevicompactum* (4), its discovery did promote intense interest into the isolation of microbial natural products, which led to the discovery of many therapeutically useful drugs and the development of many valuable isolation protocols. The original isolation of penicillin by the team headed by Florey and Chain utilized a countercurrent method (*see* Chap. 7), which was crucial to their success where others had failed (5).

2.1. The Isolation of Therapeutic Penicillin

Penicillin, rather than representing a distinct compound, is a collective term used to describe the class of compounds containing the fused bicyclic β-lactam and thiazolidine moiety (**Fig. 2**). The major penicillin produced by the Oxford team was 2-pentenyl penicillin (penicillin I or penicillin F), while alteration of the media on which the fungus was cultured in the large-scale fermentations conducted in the United States resulted in the major penicillin produced being identified as benzyl penicillin (penicillin II or penicillin G, **Fig. 2**).[†]

The instability of penicillin presented challenges to the Oxford team, which had already defeated at least two previous groups. In 1932, a group led by Raistrick grew *Penicillium notatum* and isolated from it the non-antibiotic pigment, chrysogenin (6). While the presence of penicillin was recognized, it was not isolated. Three years later in 1935, Reid published

[†] The reader is directed to the Mitsubishi Chemical corporation website, http://www.m_kagaku.co.jp/index_en.htm.

	Penicillin Name	
	British	American
R =	I	F
R =	II	G
R =	III	X
R =	IV	K

Fig. 2. The structure of some natural penicillin antibiotics isolated from *P. notatum*.

work detailing the inhibitory effects of *P. notatum*; but again the substance responsible for the activity, already named penicillin by Fleming, remained elusive ***(7,8)***. The reason for the chemical instability was the β-lactam moiety, which is susceptible to cleavage in either acidic or basic conditions. The Oxford team satisfactorily overcame this problem, producing penicillin in an active stable form through a combination of liquid–liquid and liquid–solid chromatography. It was the inventiveness of Norman Heatley, one of the team members, who developed a countercurrent extractive separation technique, that resulted in the capacity to produce large amounts of penicillin required for the in vivo experiments that ultimately resulted in the saving of countless lives and won the leaders, Chain and Florey, the 1945 Nobel prize in Physiology or Medicine.[§] The isolation protocol, represented in the text here, is taken from the seminal paper on penicillin production and summarizes the successful strategy employed ***(5)***.

1. Penicillin can be extracted by ether, amyl acetate, and certain other organic solvents from an aqueous solution whose pH has been adjusted to 2.0. From the organic solvent, the penicillin may be re-extracted by shaking with phosphate buffer or with water, the pH of which is maintained at 6.0–7.0.

[§] The 1945 Nobel prize was shared with Alexander Fleming, who while definitely not the first person to recognize the antibiotic effect of the *Penicillium* sp., was the first to demonstrate that a solution injected into an animal was not toxic, thereby laying the foundation of the efforts led by Florey and Chain in the discovery of penicillin.

2. A continuous countercurrent extraction apparatus is used. The crude penicillin having been filtered and acidified, is passed through special jets that break it up into droplets of uniform size. These are allowed to fall through a column of amyl acetate, to which the penicillin is given up (*see* **Note 1**).
3. Fresh solvent is continuously fed into the bottom of the column, from the top of which an equal amount of penicillin-rich solvent is collected for further working up.
4. Batches of 3 L each of the penicillin-containing solvent as delivered from the extraction apparatus are extracted with five successive amounts of 300 mL each of water, using baryta to adjust the pH to 6.5–7.0.
5. The strongest aqueous extract is partially decolorized by shaking with about 8% of animal charcoal and filtering.
6. The partially decolorized solution is cooled, acidified, and extracted into successive amounts of ether; the strongest of the ether extracts is then passed through an adsorption column of Brockmann alumina. The column is eluted with a phosphate buffer (pH 7.2) and the fractions containing the most penicillin are extracted back into ether.
7. Finally, the penicillin is extracted back into water using sodium hydroxide to adjust the pH (*see* **Note 2**).

The penicillin thus obtained, given that it was isolated under British culture conditions would have been mainly penicillin I (2-pentenyl penicillin, **Fig. 2**), was described as a deep reddish-orange fluid with a faint smell and a bitter taste, indicating that while used therapeutically it was not 100% pure.

2.2. The Principle of Countercurrent Chromatography

Countercurrent chromatography has its origins in liquid–liquid extraction (*see* Chap. 10), whereby two immiscible liquids are used to separate organic compounds based on their differential solubility in each solvent. The distribution of the solute molecules between the two phases is governed by the partition constant (K), which is a constant at any given temperature, and theoretically solutes possessing differing partition constants can be separated. Separation occurs in an analogous manner to solid–liquid chromatography or gas–liquid chromatography, where solute molecules are separated on the basis of an equilibrium established between the two phases involved

$$K = C_A/C_B$$

where C_A and C_B represent the concentration of the solute (S) in the two solvents.

While details on the general methodology, especially with respect to natural product isolation, can be found in Chapter 7, an outline of various aspects of countercurrent methods in relation to the isolation of microbial natural products is presented here.

2.2.1. Countercurrent Distribution (CCD)

Craig *(9)* recognized the fact that liquid–liquid extraction, for example in a separatory funnel, allowed only limited resolving power, and that the method could be extended to multiple equilibrium events thereby increasing the resolving potential. Multiple solutes could be resolved based on their respective partition constants, and he showed that this method was applicable for a wide variety of binary mixtures. Craig also demonstrated that when the ratio of partition constants between two solutes was greater than 4, separation was easily achieved but also showed that more challenging separations were feasible utilizing his methods. For example, the partial purification of α- and β-naphthoic acids, where the ratio of partition constants was only 1.08, and purification of *p*-toluic and benzoic acids, where the ratio of partition constants is 2.48, was readily achieved. Further to these experiments, Craig was able to show that simply by increasing the number of equilibrium events (transfers), he was able to dramatically raise the resolving power in a system. The separation of a mixture of C12, C14, C16, and C18 fatty acids was readily achieved by increasing the number of transfers *(10)*. Theoretically, an infinite number of transfers could be utilized to achieve a resolution given that the method does not suffer from adsorption losses, as is frequently the case in other forms of chromatography. The isolation of natural products, many of which were microbial in origin, was achieved using this countercurrent method as illustrated through the isolation of the antibiotic active component, conocandin (**Table 1**) from cultures of the fungus *Hormococcus conorum* *(11,12)*.

2.2.2. Droplet Countercurrent Chromatography (DCCC)

DCCC best resembles the countercurrent technique utilized by Heatley in the original work on the isolation of penicillin *(5)*. Whereas Heatley's equipment consisted of a single column, commercial DCCC apparatus may contain as many as 600 columns connected by capillaries through which a mobile phase is pumped. The mobile phase flow can be discretionally reversed to account for the relative density of the stationary phase solvent and the solute molecules can be added in either solvent. The method has been

Table 1
Representative Microbial Natural Products Isolated Using Countercurrent Methods

Structure/name/organism	Countercurrent method	Ref.
Conocandin *H. conorum*	CCD	(12)
Gibberellin A58 *G. fujikuroi*	DCCC	(14)
6,7-Dihydrophomopsolide B *Penicillium* sp.	HSCCC	(17)

widely used in the isolation of natural products and has been the subject of an excellent review by Hostettmann and Marston *(13)*. A multitude of natural products have been isolated, and solvent systems suitable for a wide variety of structural classes have been established, thereby lessening the

ordeal of finding a suitable solvent system. Both aqueous and nonaqueous systems have been established, allowing virtually any class of compound to be separated. Among the many microbial natural products isolated by this method are members of the gibberellin class of plant growth regulators isolated from the fungus *Gibberella fujikuroi* (**Table 1**) ***(14)***.

2.2.3. High-Speed Countercurrent Chromatography (HSCCC)

High-speed countercurrent chromatography represents the most advanced model in the evolution of countercurrent chromatography. It has overcome many of the pitfalls associated with earlier methods and allows the rapid separation of solute molecules from often complex mixtures. For this reason, it has been routinely used as a separation tool ***(15)***, and examples of its application in the isolation of natural products can be found in Chapter 7. Among the many examples where HSCCC has been used are those where the technique has been utilized in microbial natural product isolation for the separation of zaragozic acids (squalestatins) ***(16)***, phomopsolides ***(17)***, and gibberellins, to name a few (**Table 1**).

2.3. Liquid Ion Exchange Extraction

The separation of molecules has been achieved chromatographically by the partition of solute molecules between immiscible phases in a variety of countercurrent techniques. When the solute molecule possesses ionizable functionality, e.g., carboxylic acids or amines, then the possibility of separation based on ion-exchange (*see* Chap. 6) becomes a reality ***(18)***. Examples of the application of this technique in the isolation of microbial natural products, e.g., cephamycin A ***(19)***, gualamycin ***(20)***, and zaragozic acid A ***(21)***, are summarized in **Table 2**.

Another ion-exchange process useful in the isolation of microbial natural products is the method of liquid ion-exchange. The *IUPAC Compendium of Chemical Terminology* defines this process as a liquid–liquid extraction process that involves a transfer of ionic species from the extractant to the aqueous phase in exchange for ions from the aqueous phase. The method has found widespread use in the mining industry where it is used for the removal of metal ions from aqueous solution. For example, zinc has been recovered by treating aqueous solutions with organic solutions containing 2-hydroxybenzophenoneoxime and substituted 8-hydroxyquinolines. The zinc ions are transferred into the organic phase, forming an organic soluble ion pair. The organic phase is then separated and

Table 2
Representative Microbial Natural Products Isolated Using Classical Ion-Exchange Methods

Structure/name/organism	Ion-exchanger	Ref.
Cephamycin A *S. griseus*	Amberlite IRZ-68(Cl^-) and DEAE Sephadex A-25	*(19)*
Gualamycin *Streptomyces* sp., NK11687	Dowex 50W (H^+) and CM-Sephadex C-25 (Na^+)	*(20)*
Zaragozic acid A unidentified fungal culture, ATCC 20986	Dowex 1-X2 (Cl^-)	*(21)*

subsequently acidified releasing the zinc ions *(22)*. The technique, while not so widely used in drug discovery operations, has found application in the extraction of microbial natural products, where the isolation of β-lactam antibiotics from *Streptomyces olivaceus* and that of the polyene,

amphotericin B from *S. nodosus*, have been achieved and serve as examples (**Table 3**) ***(23,24)***.

The isolation of amphotericin B is notable, given that it is an antifungal drug used to treat systemic mycoses which, despite the side effects of its use, remains the drug of choice for life-threatening fungal infections. The isolation of amphotericin B is difficult because it is only sparingly soluble in organic solvents. However, the use of the liquid ion-exchange method greatly enhances its solubility presenting an elegant and efficient method, as outlined later (**Fig. 3**), for the large-scale isolation of this valuable compound.

3. Liquid–Solid Chromatography

A plethora of liquid–solid chromatographic processes have been developed and used in the isolation of natural products, be they produced microbially or otherwise, including gel permeation, adsorption, ion-exchange, and affinity chromatographic methods (*see* Chaps. 4–10). It is not desirable here to review these methods but merely to briefly explore a method used widely in microbial natural product isolation.

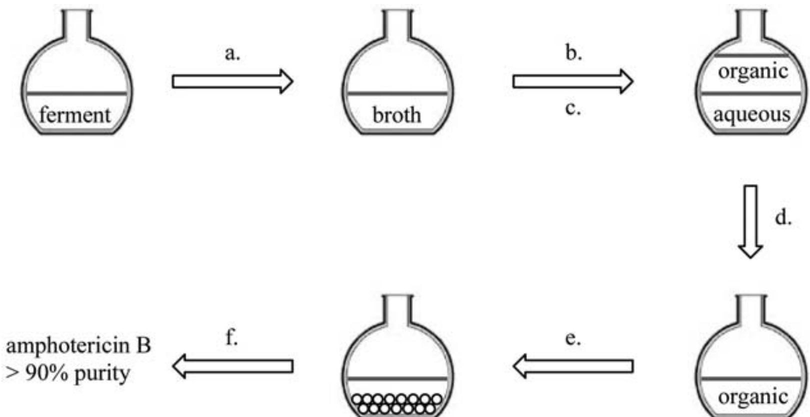
3.1. Polymeric Adsorbents

Fermentation broths are predominantly composed of water, and therefore the isolation of exocellular microbial natural products that have been exuded into the growth medium presents problems unique to the field. While intracellular metabolites are readily concentrated by mechanical removal of the cellular biomass and subsequent extraction, the enrichment of secondary metabolites present in fermentation broths requires special attention. Liquid–liquid extraction methods have proven useful in some circumstances. However, they require specialized equipment, and the use and recovery of large volumes of organic solvents, imposing adverse economic and environmental impacts on an operation. As such, alternative strategies have been sought to address these issues, and liquid–solid chromatographic processes have been developed resulting in the creation of a variety of synthetic polymeric adsorbents that have a high affinity for organic molecules.

Polymeric adsorbents represent a large group of available products, including resins supplied by the Mitsubishi Chemical Corporation and the XAD range of Amberlite resins produced by the Rohm and Haas

Table 3
Representative Microbial Natural Products Isolated Using Liquid Ion-Exchange Methods

Structure/name/organism	Organic phase/ion-exchanger	Ref.
MM 4550, *S. olivaceus*	CH_2Cl_2	(23)
MM 17880, *S. olivaceus*		
Amphotericin B *S. nodosus*	BuOH Aliquat 336	(24)



Key steps involved:

a) the fermentation is filtered and the broth retained.

b) pH is adjusted to 10.5 using 7% NaOH solution.

c) broth is mixed with 1-butanol containing 7% w/v aliquat 336.

d) phases are separated and the organic phase is retained. At this stage amphotericin content can be estimated by monitoring at 405 nm.

e) 1% v/v EtOAc is added resulting is gradual hydrolysis of ethyl acetate by aqueous alkaline solution dissolved in the organic phase. The subsequent lowering of the pH (as neutrality is approached hydrolysis stops) results in precipitation of amphotericin B as spherulites (10-40 μm in diameter).

f) filtration recovers over 70% of available amphotericin B obtained in over 90% purity as determined by NMR and HPLC.

Fig. 3. Isolation procedure for amphotericin B using the liquid ion-exchange protocol.

company.[¶] While each of the resins has its own special characters and suggested applications, the production of resins is based on similar technology. A variety of materials are used to manufacture the resins, with the most common polymeric supports for use in fermentation applications being based on crosslinked polystyrene matrices that are referred to as aromatic resins. Other chemical structures for synthetic adsorbents are

[¶]The reader is directed to the Mitsubishi Chemical Corporation website, http://www.m-kagaku.co.jp/index_en.htm.

modified aromatic resins, e.g., SP207 and methacrylic resins such as HP2MG (Table 4). The adsorbents are presented as spherical particles characterized by large surface areas. Surface areas of up to 1000 m^2/g are common and possess a highly porous structure. This combination of high surface area and porous nature of the material allows a high uptake of organic solute molecules from aqueous solutions, such as those encountered in fermentations. Owing to the author's experience with diaion products, they are reviewed here. The Amberlite series of XAD resins have similar applications, and a visit to the Rohm and Haas website provides information to the reader with regard to the range and breadth of application of these materials.

Pore size is an important parameter that determines the adsorptive characteristics, and as such resins are provided with varying pore structures. A molecular sieving effect can be achieved by using resins with small pores, thereby favoring adsorption of small molecules while larger ones are excluded. For example, Diaion HP20 is recommended for the adsorption of solute molecules with molecular weights less than 20–30 kDa, while Sepabeads SP825 possessing a smaller pore radius is recommended for the adsorption of solute molecules with a molecular weight less than 1 kDa. If the nature of the solute molecule is known, the most appropriate type of adsorbent can be selected according to the pore size of adsorbent. While in the initial isolation of a natural product this is seldom the case, repeat isolations when the target molecule has been identified can be greatly accelerated and simplified by judicious selection of the most appropriate resin.

The concentration of organic solutes is achieved using these resins by either chromatographic filtration of the fermentation broth, or the polymeric adsorbent can be added to the broth and the resulting suspension stirred gently to allow adsorption. In either case, the fermentations should have been treated to remove cellular biomass prior to adsorbent application to avoid physical impediments to flow. In the author's laboratories, broth is delivered to a column of resin by peristaltic pumping, ensuring a constant flow rate thereby allowing adequate time for diffusion of the solute molecules into the porous support. Typically, a flow rate of 5 bed volumes per hour is employed for the loading of resins, although the raffinate should be monitored to ensure retention of the solute molecules. Once adsorbed, solute molecules are desorbed by applying a suitable concentration gradient to the resin. The synthetic adsorbents are stable in acidic and alkaline solutions and most organic solvents, and they can be easily

Table 4
Properties of Selected Resins From Mitsubishi Chemical Corporation

Resin	Diaion HP20	Diaion HP21	Sepabeads SP825	Sepabeads SP207	Diaion HP2MG
Partial structure					
Category		Aromatic		Modified aromatic	Methacrylic
apparent density (g/L-R)	680	625	690	780	720
Suggested use	Natural product isolations, extraction of antibiotics from fermentation broths, separation of peptides			Suitable for adsorption of organic solutes at low concentration or of highly hydrophilic substances. Hydrophobic resin	This type of adsorbent is suitable for adsorption of polyphenols and surfactants. Relatively hydrophilic resin
Particle size (>250 μm)		>90%			> 95%
Surface area (m^2/g)	600	570	1000	600	500
Pore volume (mL/g)	1.3	1.1	1.4	1.3	1.2
Pore radius (μm)	>20	8	5.7	11	20

Table has been produced using data from the Mitsubishi Chemical Corporation website (see **Footnote ¶**).

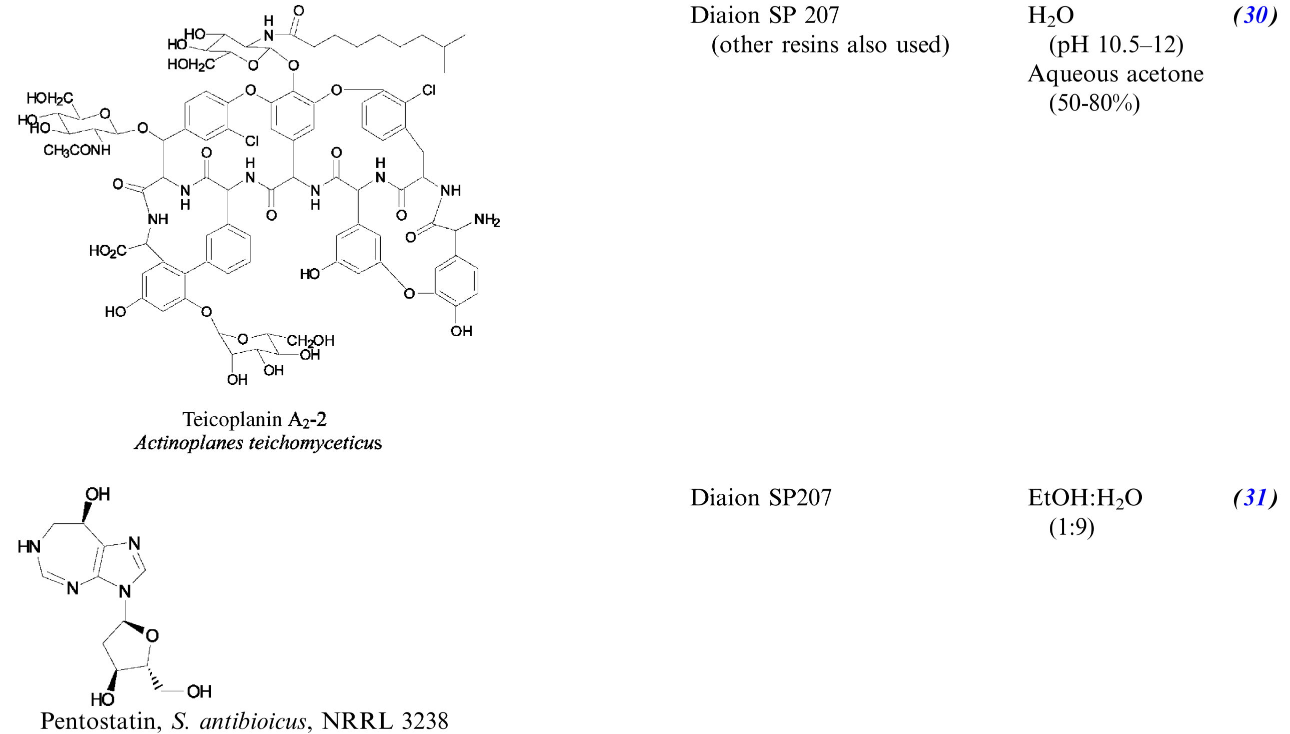
Table 5
Microbial Natural Products Where Synthetic Adsorbents Have Been Utilized in the Isolation Process

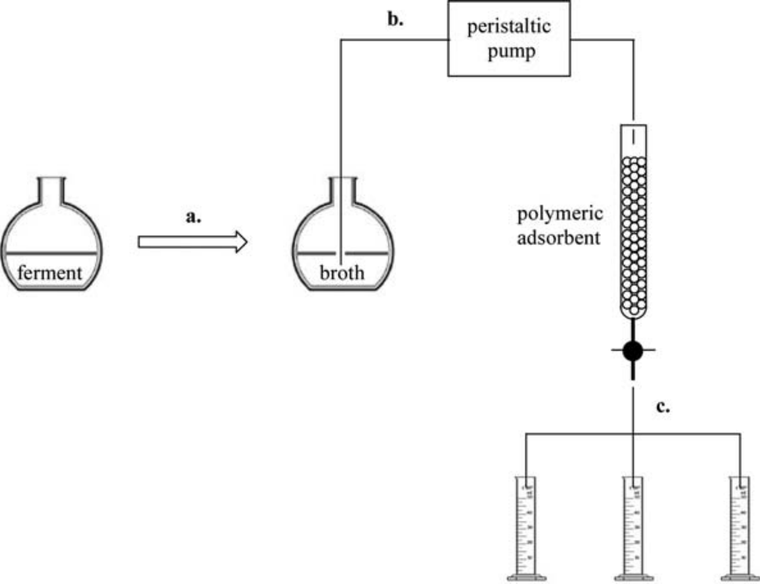
Structure/name/organism	Resin	Eluting solvent	Reference
BE-31405, *Penicillium minioluteum*	Diaion HP-20	Aqueous MeOH (50-100% in MeOH)	(25)
Calphostin D, *Cladosporium cladosporioides*	Diaion HP-20SS	Aqueous MeOH (90-95% in MeOH)	(26)

(*Continued*)

Table 5
Microbial Natural Products Where Synthetic Adsorbents Have Been Utilized in the Isolation Process (*Continued*)

Structure/name/organism	Resin	Eluting solvent	Reference
Tubelactomicin A, *Nocardia sp.*	Diaion HP-20	50% aqueous MeOH 50% aqueous acetone	(27)
DC-52 DX-52-1 (artifact) *S. melanovinaceus*	Diaion, SP-207 HP-10	6% aqueous acetone	(28) (29)

 Teicoplanin A_2-2 *Actinoplanes teichomyceticus*	Diaion SP 207 (other resins also used)	H_2O (pH 10.5–12) Aqueous acetone (50-80%)	*(30)*
Pentostatin, *S. antibioicus*, NRRL 3238	Diaion SP207	$EtOH:H_2O$ (1:9)	*(31)*



Key steps involved:

a) the fermentation is filtered and the broth retained while the mycelial matt which also contains the target compounds is extracted in EtOH:CH_2Cl_2 (9:1)

b) peristaltic pumping delivers broth at a flow rate of ~5 bed volumes/hr.

c) column was eluted at 1.8 bed volumes per hour using a step gradient from H_2O (fraction discarded), 50% MeOH/H_2O, 100% MeOH and a final elution with acetone (this fraction contained target compounds). Each fraction represented ~1.5 bed volumes.

Fig. 4. Partial purification of ustilaginoidin antibiotics from a cultured entomogenous fungus.

regenerated under mild conditions for repeated use. The aromatic adsorbents are most frequently desorbed by applying an increasing methanol gradient at a flow rate of 1–2 bed volumes per hour and, if necessary, a final elution with acetone will generally ensure complete removal of solute molecules. In the case of ionizable functional groups existing in the solute molecules, solutions of weak acids or bases can be utilized to ensure

Fig. 5. Structure of ustilaginoidin D isolated from an unidentified entomogenous fungus.

conversion to the ionized species, which then allows rapid elution owing to the repulsive interactions with the support. Examples of isolations where synthetic resins have been utilized in the isolation process and the solvents used to effect elution from the resins are shown for BE-31405 ***(25)***, calphostin D ***(26)***, tubelactomicin A ***(27)***, DC-52 and the artifact derived from it DX-52-1 ***(28,29)***, teicoplanin A_2-2 ***(30)***, and pentostatin ***(31)*** in **Table 5**.

By way of example, the isolation of a series of ustilaginoidins obtained from an (Metarhizium anisopliae) entomogenous fungi has been optimized recently in the author's laboratory ***(32)***. The filtered broth (10 L) was applied to a column of Diaion SP207 (5 cm i.d $\times$ 22 cm) at a rate of approximately 2 L per hour. Elution as indicated in Fig. 4 was carried out and the fractions assayed by biological activity and NMR, which subsequently showed that the acetone eluate was composed entirely of a mixture of three ustilaginoidins (>95% of a mixture of three compounds by NMR), including the known compound ustilaginoidin D (**Fig. 5**) and two previously unreported compounds. Final resolution of these three components was achieved by reversed-phase HPLC.

4. Notes

1. Acidifying the cooled broth containing the penicillin seconds before it was added to the countercurrent setup elegantly solved the problem of instability encountered in the acidification step. Extraction into amyl acetate then meant exposure to acid was of the order of seconds.
2. As the solution is not buffered, maximum care must be taken when adding the alkali, as penicillin is rapidly destroyed in alkaline solution.

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