CHROMATOGRAPHIC APPROACH TO POLAR COMPOUNDS: ISOLATION OF HYDROPHILIC CONSTITUENTS OF THE MARINE SPONGE *CRAMBE CRAMBE*

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The separation steps utilized for the isolation of the constituents of the marine sponge *Crambe crambe* polar extracts are discussed in detail.

Keywords: marine sponge *Crambe crambe*; chromatography.

The isolation of secondary metabolites always present a crucial question: the choice of the more adequate chromatographic procedure(s) to be utilized, as a consequence of the nature of the compound(s) to be separated[1]. In recent years, many chromatographic techniques have been developed with the aim of facilitating natural products isolation, and much attention has been given to the search of chromatographic techniques that allows the isolation of unstable compounds.

Some authors have discussed different approaches for the isolation of hydrophilic compounds. Shimizu[2] has suggested the utilisation of ion-exchange resins and gel filtration for the isolation of marine bioactive polar compounds. Blunt[3] utilized large scale reverse-phase chromatography for the same purpose. Quinn[4] has discussed the utility of adsorption chromatography on XAD-type polymers for desalting extracts and subsequently separation of hydrophilic and lipophilic compounds. Finally, Cardellina[5] has presented remarkable results of chromatographic separation of *Lyngbya majuscula* constituents on Sephadex LH-20. Hence, the choice of a particular technique is not trivial, although extremely important, as stated by Bruening *et al.*[6].

Our work on a polar extract from the marine sponge *Crambe crambe* has involved many different chromatographic separations[7-9], which proved to be useful in each step of purification of this complex extract. Here we will discuss, in some extension, the separation scheme which was followed for the isolation of the constituens of the n-butanol extract of *Crambe crambe*.

The methanol stored sponge was exhaustively extracted with a methanol/dichloromethane 1:1 mixture. After evaporation of dichloromethane, the extract was partitioned as showed in figure 1. The hexane soluble fraction was not analysed by us, but Cafieri *et al.*[10] have studied the sterol composition of *Crambe crambe*. The aqueous fraction presented a high quantity of inorganic salts, and it was discarded.

The CCl_4 fraction presented little ichthyotoxicity against the fresh water fish *Lebistes reticulatus*, and the n-butanol one has shown most of this activity. A TLC screening was then envisaged in order to efficiently monitoring each separation step. TLC analyses of the CCl_4 and n-butanol extracts have shown that they have similar constituents. The eluents utilized for the TLC screening were: CH_2Cl_2/MeOH/AcOH 8:2:0.1, n-BuOH/AcOH/H_2O 4:1:1, CH_2Cl_2/MeOH/NH_4OH 25% 8:2:0.1; and the TLC reagents: anisaldehyde-sulfuric acid (oxidizing agent), vanilline-sulfuric acid (oxidizing agent), Sakaguchi (α-naphtol/sodium hypobromide, for guanidine, urea and thiourea derivatives), sodium nitroprusside-potassium

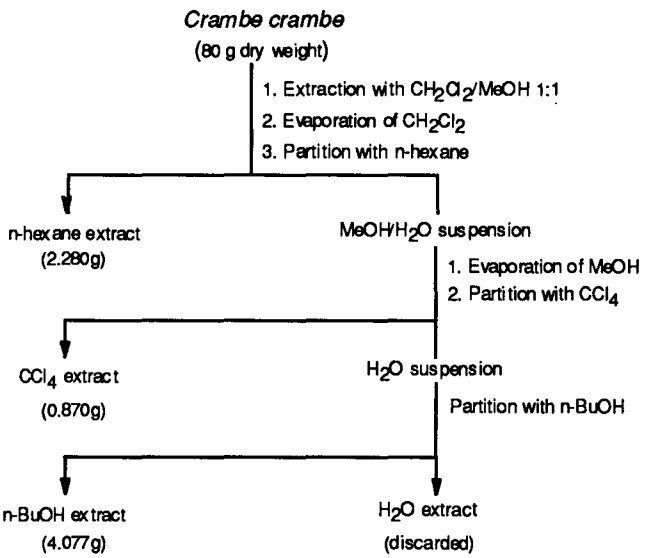
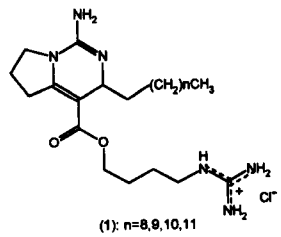


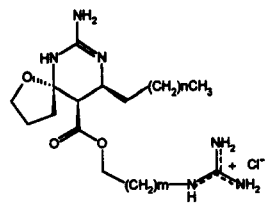
Figure 1

hexacyanoferride (for guanidine, urea and thiourea derivatives), Dragendorff (alkaloids) and ultra-violet irradiation. Some compounds with intermediate polarity were present in both n-butanol and in CCl_4 fractions. After their isolation they were identified as crambescin A (**1**), crambescin B (**2**), crambescin C1 (**3**) and their respective homologues[7,8]. The structures of crambescin B (**2**, n=9) and crambescin C1 (**3**, n=9) major homologues were recently revised by Jares-Erijman *et al.*[11] and by Snider and Shi[23] to (**2a**) and (**3a**) by tandem mass spectrometry (fabms/cid/ms) experiments and synthesis, respectively.

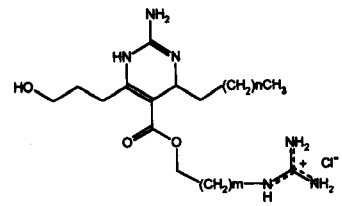
The n-butanol fraction was highly ichthyotoxic against *Lebistes reticulatus*. It was a very complex fraction, with more than 30 different TLC spot-visualised compounds, which presented a wide range of polarity. The less polar components migrate well with CH_2Cl_2/MeOH 9:1 as eluent, and they were identified as adenine (**4**), 2-desoxy-adenosine (**5**), thymidine (**6**) and an homologue mixture of ptiloceramide(s) (**7**)[12]. Crambescins A, B and C1 and their homologues have intermediate polarity (crambescin B: rf 0.5; crambescin C1: rf 0.4; crambescin A: rf 0.30 in CH_2Cl_2/MeOH/AcOH 8:2:0.1, stained



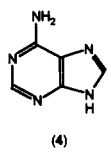
(1): n=8,9,10,11



(2): n=8,9,10,11; m=4
(2a): n=7; m=6



(3): n=8,9,10,11; m=4
(3a):n=7; m=6



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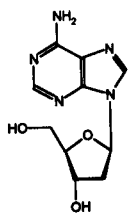

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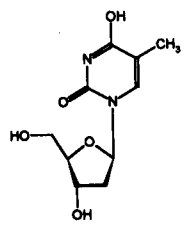

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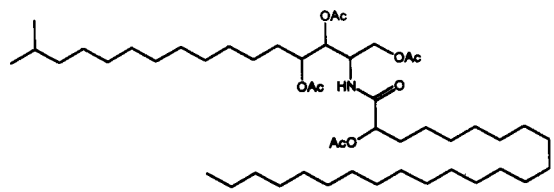
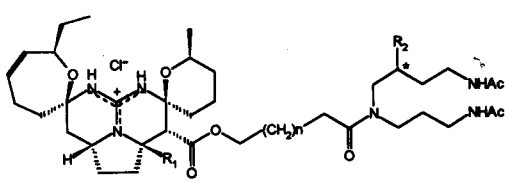
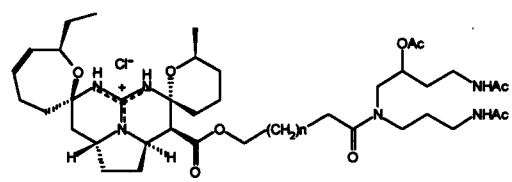


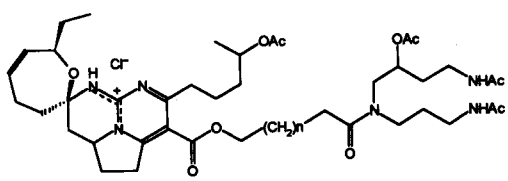
(7)



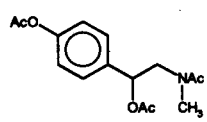
(8): R_1=H, R_2=OAc, n=13
(10): R_1=OH, R_2=OAc, n=13, °(S)
(16): R_1=OH, R_2=OH, n=14
(17): R_1=OH, R_2=OH, n=15
(18): R_1=H, R_2=H, n=13



(9): n=13



(11): n=13



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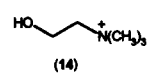

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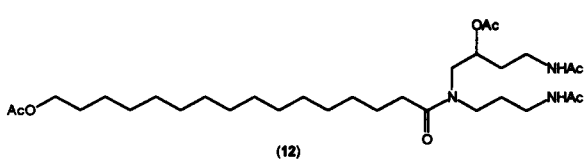
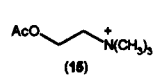


(12)



(15)

with vanilline-sulfuric acid reagent after heating at 100°C for 3 minutes). Moreover, these compounds "slip" over TLC in almost any eluent, and this was troublesome for their separation on silica-gel. Finally the more polar compounds of the *Crambe crambe* n-butanol extract have shown to be primary amines, as concluded from their intense violet and brown reponse to ninhydrin reagent (TLC eluent: EtOH/NH_4OH 25% 7:3).

Initial steps for the separation of these polar compounds have involved chromatographies on Sephadex LH-20 which is a rather inert stationary phase, widely utilized for the separation of polar and/or unstable compounds. The chromatography of C. *crambe* n-butanol extract on Sephadex LH-20 with 5:1 $CHCl_3$/MeOH as eluent gave poor separation results and solvent density makes Sephadex LH-20 floating in these conditions. In spite of this, we were able to isolate minute quantities of impure crambescin C1 and α-O-methyl-glucose. Further chromatography of the n-butanol extract on Sephadex LH-20 with methanol as eluent led us to obtain two fractions: the first one with the more polar, basic ninhydrin positive compounds (crambescidins 816, 800, iso-800, crambidine A) and the second with less polar components (the homologue mixture of crambescins A, B and C1, ptiloceramide and the above mentioned nucleoside derivatives). Further chromatography of the second fraction on Sephadex LH-20 with CH_2Cl_2/MeOH 1:1 led us to obtain five main fractions (see figure 2):

a) the first one was constituted by the very polar, basic ninhydrin positive compounds.

b) the second has mainly one compound. After purification by silica-gel "flash" chromatography[13], followed by acetylation and purification, this compound was identified as an homologue mixture of ptiloceramide(s) (**7**, major homologue).

c) the third fraction contained the compounds giving a positive Sakaguchi test and was constituted by the homologue mixture of crambescins A (**1**), B (**2a**) and C1 (**3a**).

d) the fourth fraction presented one predominant compound which, after purification by silica-gel flash chromatography, followed by acetylation and further silica-gel flash chromatography purification, was identified as peracetyl α-O-Me-glucose.

e) and the last fraction was constitued by UV absorbing compounds. These, after separation by silica-gel flash chromatography and purification by C-8 and C-18 reverse-phase HPLC, were identified as adenine (**4**), 2'-desoxy-adenosine (**5**) and thymidine (**6**).

Further steps were attempts to separate the respective homologue mixtures of crambescins A (**1**), B (**2a**) and C1 (**3a**). Droplet counter current chromatography of this fraction with $CHCl_3$/MeOH/n-BuOH/H_2O 10:10:1:6 in descending mode could eliminate the less polar impurities, but the Sakaguchi positive compounds did not migrate in these conditions. The fraction was then chromatographied in the ascending mode with the same eluent, giving a first polar fraction with basic ninhydrin compounds separated from a second fraction constituted by the Sakaguchi positive ones. A number of unsuccessful separation trials (which gave poor or no separation) were realized with the following stationary phases: large-scale reverse-phase chromatography (with a MeOH gradient in H_2O as eluent)[3], strong acid ion-exchange chromatography (with Amberlite IR-120 and 0.01-0.05N NH_4OH gradient as eluent), weak acid ion-exchange chromatography (with BIO-REX 70 and 0.01-0.05N NH_4OH gradient as eluent), Florisil (gradient of MeOH in AcOEt) and neutral alumine (MeOH).

The partial separation of the crambescins mixture was achieved by silica-gel flash chromatography with a gradient of methanol in dichloromethane with 0.5% of trifluoroacetic acid as eluent. We obtained an homologue mixture of crambescin A (**1**), as well as an homologue mixture of crambescin B (**2a**), but we were unable to get the homologue mixture of crambescin C1 (**3a**). It was also verified an increase in the weight of each fraction isolated, as we can observe in scheme 2. Nevertheless, careful RMN-^{1}H, RMN-^{13}C and IR analysis of the partially purified crambescins A and B did not indicate any contaminant. It was thus supposed the presence of an inorganic impurity. The homologue mixture of crambescin B was then purified by silica-gel flash chromatography with a gradient of AcOEt/MeOH 1:1 in CH_2Cl_2. As we observed crambescin B degradation in a considerable extent (see its weight lost in scheme 2), crambescin A was only filtered on IRA-400 ion-exchange resin in order to eliminate inorganic impurities.

The homologue mixture of crambescin C1 (**3a**) was very difficult to obtain in pure state. It was always contaminated with crambescin B (**2a**) and its homologues, and we speculated the possibility of interconversion between these two compounds (and respective homologues) *via* an acid or base catalysed intramolecular cyclisation of crambescin C1 into crambescin B[14]. Many tentatives of crambescin C1 derivatization by acetylation or reaction with pentane-2,4-dione were tried, but only intractable mixtures were obtained[8]. Tentatives of cyclization of crambescin C1 into crambescin B were also carried out in basic (pyridine, Na_2CO_3) and acid conditions (AcOH in dioxane), all unsuccesfully.

We tried to separate crambescins B and C1 and their respective homologues by increasing the chromatographic trajectory. Thus, we linked four columns in a 2.7 meters descending-ascending-descending-ascending trajectory using Sephadex LH-20 as stationary phase and methanol as eluent. We were able to obtain the homologue mixture of crambescin A separated from the homologue mixture of crambescin B and the homologue mixture of crambescin C1, but no separation between these two last "families" of compounds. Curiously, the light and more polar (on TLC) crambescin A has a shortest elution volume than crambescins B and C1 (more heavy and less polar) on Sephadex LH-20 in these conditions, in contrast to the results discussed by Cardellina for *Lyngbya majuscula* secondary metabolites[5].

Literature mentions that polyvinylpolypyrrolidone (PVPP) could separate isomeric alcohols with excellent results[15-19] led us to use this stationary phase for crambescin C1 isolation. The ability of PVPP to separate alcohols result from hydrogen bonding formation between the lactam carbonyl of stationary phase and the hydrogen of the alcohol function(s) from the compound to separate. Hence, the separation process in PVPP is function of alcohol acidity. Chromatography of the mixture of crambescin B and crambescin C1 respective homologue "families" on PVPP furnished the homologue mixture of crambescin C1 free from the homologue mixture of crambescin B. The homologue mixture of crambescin C1 was finally purified by gel permeation on Sephadex LH-20 using methanol as eluent. No interconversion between crambescin C1 and crambescin B could be observed at room temperature for several weeks.

The heaviest fraction containing the strong polar, basic ninhydrin positive compounds was chromatographied on Sephadex LH-20 "long trajectory" with methanol as eluent. We could obtain six complex fractions, constituted by more than 20 different ninhydrin positive compounds. These later were chromatographied on BIO-GEL P2 with H_2O/EtOH 8:2 as eluent. BIO-GEL P2 is a polyacrylamide polymer, recomended for the isolation of polypeptides lighter than 2.500 daltons. It was utilized for the separation of the strongly basic saxitoxin and its derivatives[20]. After chromatography of the six basic ninhydrin positive fractions on BIO-GEL P2, we obtained a variety of new fractions, which were separately acetylated and purified by silica-gel flash chromatography using two eluents alternatively: a gradient of methanol in dichloromethane and a gradient of $CHCl_3$/MeOH/H_2O/i-PrOH

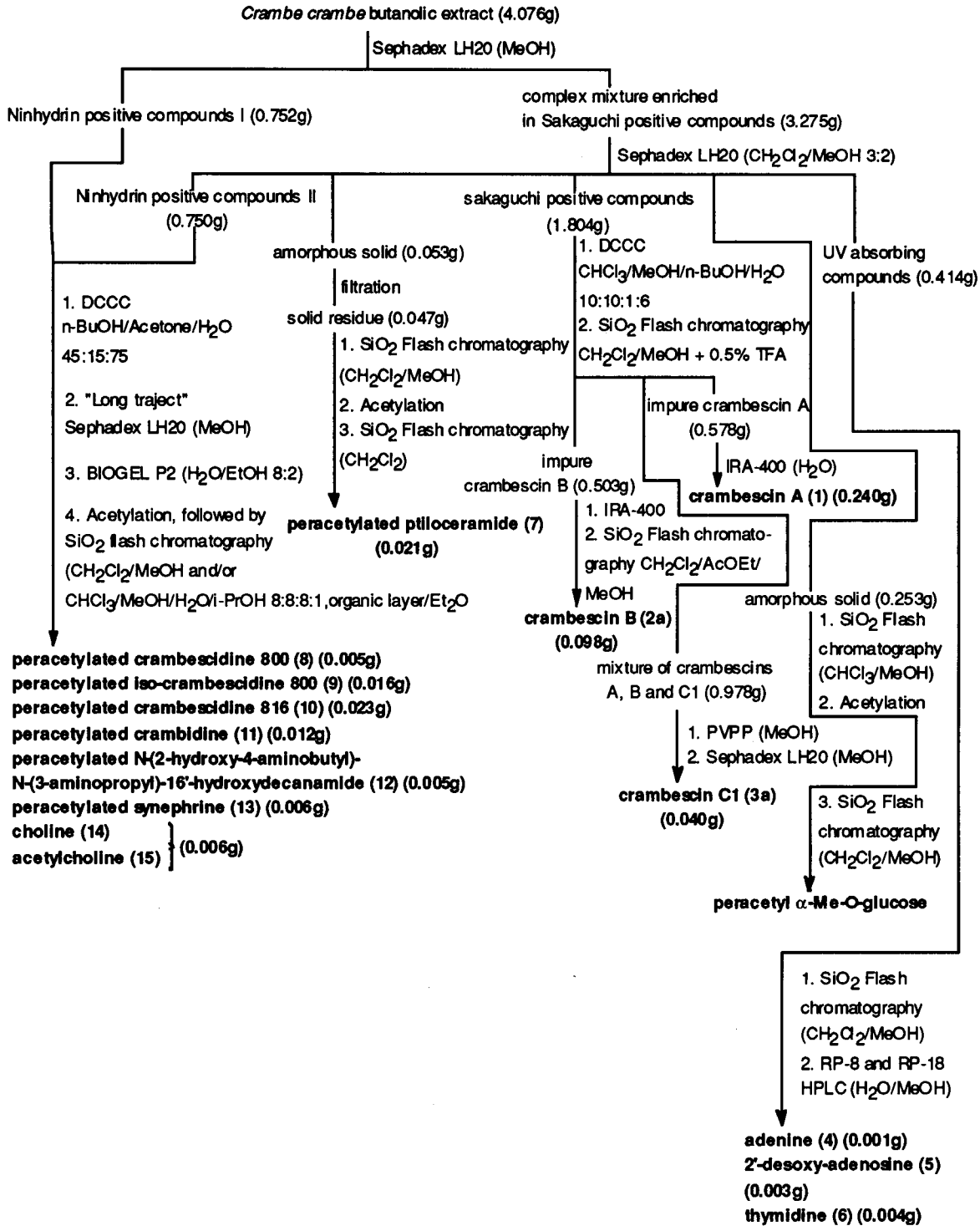


Figure 2

8:8:8:1 (organic layer) in ethylic ether. This last eluent has shown an excellent separation power in silica-gel flash chromatography, and may be considered as an alternative for the separation of relatively polar compounds. This eluent allowed the isolation of an isomer of peracetyl crambescidine 800 (**8**), namely peracetyl iso-crambescidine 800 (**9**) [9], which possess litlle diference (Δ rf less than 0.1) in retention time on TLC over silica-gel. Other compounds isolated from these basic fractions after acetylation and purification were peracetyl crambescidine 816 (**10**), peracetyl crambidine A (**11**), peracetyl N-(2-hydroxy-4-aminobutyl)-N-(3-aminopropyl)-16'-hydroxyhexadecanamide (**12**), peracetyl synephrine (**13**), choline (**14**) and acetylcholine (**15**) [9].

Other authors[21] have isolated the crambescidines 800 (**8**, R_2=OH), 816 (**10**, R_2=OH), 830 (**16**) and 844 (**17**) from *Crambe crambe* by Sephadex LH-20 (MeOH as eluent) gel filtration of $CHCl_3$ extract, followed by partition with hexane-EtOAc-MeOH-H_2O (4:7:4:3) and purification by HPLC with

a cyano-derivatized column. Kashman *et al.*[22] isolated a closely related compound, ptilomycaline A (**18**), from the sponges *Ptilocaulis spiculifer* and *Hemimycale* sp., by chromatography of the crude extracts on an NS-gel column (Nippon Seimitsu Kagaku 10503). These different approaches to closely related problems indicate that the choice of chromatographic methods for the isolation of natural products is far from being an obvious one-way process. While Jares-Erijman[21] and Kashman[22] bioassay guided procedures are more direct and led efficiently to biologically active products, our complex isolation procedure showed that a large array of compounds can be isolated by a combination of different chromatographic procedures.

Thereafter, a general plan may be envisaged for the isolation of polar unknown natural products (in agreement with most of marine natural products literature). First, it is preferable to choose rather inert and mild stationary phases for initial fractionations of polar organic extracts. Hence, Sephadex type gels (LH-20, G-10, G-15 and G-25) and Amberlite XAD-type adsorbing resins (XAD-2, XAD-4 and XAD-7) may be commonly used. Subsequent separation work may be carried by counter-current distribution (by droplet counter-current chromatography or related techniques[1]), gel filtration in better resolving (and more expensive) gels (such as BIO-GEL P2, CM-Sephadex C-25, japanese Toyopearl gels and so on), ion-exchange chromatography (in acidic or alkaline resins, dependind the nature of the compounds to separate), among some other techniques. Finally, final purification may be achieved by well known silica-gel flash chromatography for stable and not very polar compounds or by reverse phase HPLC in semi-preparative or preparative columns. Derivatization of crude mixtures is an alternative that may be envisaged, but it is in general avoided due to the imprevisible behavior of unknown compounds face to reaction conditions. It is more generally employed in final separation steps, when the nature of the compounds is better known.

Finally, as it has been stated, "... no structural studies can be carried out unless the factor is isolated in a pure state. Isolation and purification of bioactive factors are thus the first obstacles to be overcome; it frequently is the dividing point between successful studies of further structured-based investigations on mode of action, and so on."[6].

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