ISOLATION, PURIFICATION AND PROPERTIES OF THE HEXAENE MACROLIDES CANDIHEXIN I AND CANDIHEXIN II

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The hexaene macrolide candihexin produced by the mutant 18A2 of *Streptomyces viridoflavus* IMRU 3685 has been found to be a complex of two component fractions which we have named candihexins I and II. Extraction and purification methods have been developed for preparation of pure candihexin complex. Separation of the component fractions has been achieved by solvent fractionation and partition column chromatography. Candihexins I and II differ in solubility in organic solvents but have similar UV spectra. Candihexin I is active *in vitro* against yeasts and filamentous fungi with minimal inhibitory concentrations of 1~6 μg/ml. Candihexin II is essentially inactive.

The hexaene macrolide candihexin is produced by the colorless mutant 18A2 of the yellow candidin-producer *Streptomyces viridoflavus* IMRU 3685, isolated in this laboratory.[1] It is the first polyene isolated from a mutant streptomycete that differs from the product of the parent strain. Mutants of other polyene-producing streptomycetes have been reported which produce the same polyene complex as the parent strain but with different proportions of individual components[2]. Preliminary studies suggested a possible relationship between the components of the parent strain product, the heptaene candidin, and some of the components of the candihexin complex. It was therefore hypothesized that the mutation process has resulted in a change in the enzymatic subunits of the polyene synthetase system which control the length of the polyketide chain and the degree of unsaturation[3]. This hypothesis prompted us to study the components of the candihexin complex and their possible structural relationships with those of the candidin complex. This report describes a preliminary study of the extraction, purification, fractionation and antifungal properties of the candihexin complex. The physicochemical and biological properties of the components of the candihexin complex will be reported in following papers.

Obtaining polyene antibiotics in a pure form is a difficult process because of the unstable and complex nature of these antibiotics. Although it has been generally agreed that most polyene antifungal agents are associated with the mycelium of producing streptomycetes[4], there were early reports that in some cases considerable amounts of polyene may appear "dissolved" in the broth owing to the presence of concurrently produced dispersing agents[5]. About 80% of candihexin is found in the supernatant broth, released from the mycelium, at all stages of the fermentation[6]. This unusual distribution of candihexin made necessary a detailed study of the recovery process in order to obtain high purity product for subsequent chemical and biological studies.

Materials and Methods

Production of candihexin.

Studies on candihexin production were done in shake flasks and 50-liter fermentors. Large-scale production was carried out in a 1,200-liter fermentor containing 500 liters of medium. Medium composition, inoculum development, physical conditions, and biochemical characteristics of the fermentations were as reported before[6]. Candihexin was determined spectrophotometrically as described previously[6]. An $E^{1\%}_{1cm}$ value of 1000 for the pure product was used.

Extraction studies.

Twenty five ml aliquots of broth, supernatant or the mycelial cake from the same volume of broth, were extracted with different volumes of reagent grade *n*-butanol or chloroform-methanol (1:1, v/v) in 250 ml flasks by shaking at 250 r.p.m. for 1 hour at 28°C. After centrifugation at 4,200 r.p.m. the candihexin was determined in the upper phase by spectrophotometry. Methanol extraction of the mycelial cake was accomplished in a similar way.

To study the effect of preacidification on the proportion of candihexin recovered from mycelial cake, the culture broth was acidified to pH 5.0 with 1 N sulfuric acid before centrifugation. After separation of the mycelial cake from the supernatant, both fractions were adjusted to pH 6.5 (butanol extraction) or to 8.0 (chloroform-methanol extraction).

Counter-current distribution.

Counter-current distribution (CCD) of the candihexin complex was carried out in a 200-tube CRAIG machine using the solvent systems I: chloroform-methanol-0.025 M borate buffer (2:2:1, v/v/v) pH 8.3 or II: chloroform-methanol-0.025 M citrate-phosphate buffer (2:2:1, v/v/v) pH 6.25. Fifteen to twenty five grams of crude candihexin were introduced and developed for 550 transfers in each rum. Optical densities of upper and lower phases were measured at 379 and 405 nm for hexaene and heptaene determinations. CCD fractions were tested for antifungal activity by the agar plate diffusion method using *Saccharomyces cerevisiae* ATCC 9763 as test organism.

Purification of the candihexin complex.

I. Solvent fractionation: Sixty-eight grams of a 40 %-crude candihexin preparation were dissolved in 800 ml of 3 % methanolic calcium chloride at room temperature and filtered. Candihexin was precipitated from the vacuum-concentrated methanolic solution in distilled water. The precipitate was repeatedly extracted with water-saturated butanol pH 6.5. The resulting extract was washed several times with water and concentrated under reduced pressure until all the water was removed. The candihexin precipitate obtained from the dry butanol solution at 4°C, was collected by centrifugation, washed with diethyl ether and dried over phophorus pentoxide.

II. Partition column chromatography (CC) of semipurified candihexin was carried out on silicic acid ($SiO_2 \cdot xH_2O$, 100 mesh, Mallinckrodt) analytical (10×245 mm) and preparative (90×8 cm) columns. Samples of 100 mg (analytical columns) and 5.2 g (preparative columns) of semipurified candihexin ($E^{1\%}_{1cm}$ 675~771) were applied to the columns in the solvent system I (lower phase) which was also used to develop the columns at an approximate rate of 0.5 ml/min. in analytical and 2.0 ml/min. in preparative columns. Eluted fractions were diluted in methanol and scanned in a Cary 114 recording spectrophotometer.

Separation of candihexins I and II.

Five microgram samples of candihexin were spotted on precoated Silicagel G 250 μm plates (Analtech, Inc.). Plates were developed in acetone-water 80:20 (system III) v/v at 25°C, and scanned at 380 nm in a SD 3000 spectrodensitometer equipped with a SDC 300 density computer (Schoeffel Instrument Co.). Preparative separation of candihexins I and II was achieved on silicic acid columns equilibrated with dry acetone. The sample was applied in 80 % acetone and a discontinuous gradient of acetone-water mixtures of increasing polarity was used to develop the column.

Assay of antifungal activity of candihexins I and II.

Agar dilution tests for antifungal activity were carried out as described by LECHEVALIER *et al.*[7] Bioassays were also carried out after separation of the fractions by TLC by covering the plates with a *Saccharomyces cerevisiae*-seeded agar layer. Plates were held for 4 hours at 4°C to allow for antibiotic diffusion and incubated for 36 hours at 28°C.

Results

Extraction Studies

Results of the extraction of candihexin from culture broth, supernatant and mycelial cake with butanol or methanol with different solvent/broth ratios are given in Table 1. Extractions with butanol and methanol gave good recoveries when a volume of solvent equal to the volume of sample broth was used, but poor extractions were achieved with lower solvent/broth ratios. Results of chloroform-methanol extractions are summarized in Table 2. The amount of candihexin extracted with chloroform-methanol (chloroform-methanol-broth ratio of 2:2:1,

Table 1. Extraction of candihexin with different solvent systems (μg/ml of culture)*

Solvent/broth ratio	Whole culture	Supernatant broth	Mycelial cake
Butanol-broth, 1:1 (v/v)	338	152	188
Butanol-broth, 1:2 (v/v)	336	142	166
Butanol-broth, 1:4 (v/v)	310	140	164
Butanol-broth, 1:9 (v/v)	230	101	140
Dry butanol 1 V**			186
Methanol 1 V**			180
Methanol 0.5 V**			145
Methanol 0.2 V**			63

* Shake flask culture broth was used. Results are the average of three extraction experiments. pH during extraction 6.5.

** Dry butanol and methanol extractions were carried out directly in the organic solvent without suspending the mycelial cake in water. V refers to the volume of sample broth. No extraction of whole broth or supernatant broth is possible because of the miscibility of methanol in water.

Table 2. Comparative extraction with butanol and chloroform-methanol (1:1, v/v). Effect of pre-acidification of the broth on the distribution of candihexin*

Extraction conditions	Candihexin whole culture (μg/ml)	Candihexin supernatant broth		Candihexin mycelial cake	
		(μg/ml)	(% total)	(μg/ml)	(% total)
Butanol-broth, 1:1 (v/v)	414	285	68.8	141	34.0
**Butanol-broth, 1:1 (v/v) pre-acidified to pH 5	416	165	39.6	241	58.0
Chloroform-methanol-broth, 2:2:1 (v/v/v)	650	381	58.6	218	33.5
**Chloroform-methanol-broth 2:2:1 (v/v/v), preacidified to pH 5	569	234	41.1	359	63.1

* Fermentor culture broth was used. Average of three determinations. pH during chloroform-methanol extraction 8.0. Candihexin extracted with chloroform-methanol includes both the upper and lower phase extracts.

** In pre-acidified extractions, the whole broth, supernatant broth and mycelial cake were adjusted to pH 6.5 (butanol) or 8.0 (chloroform-methanol) before solvent extraction.

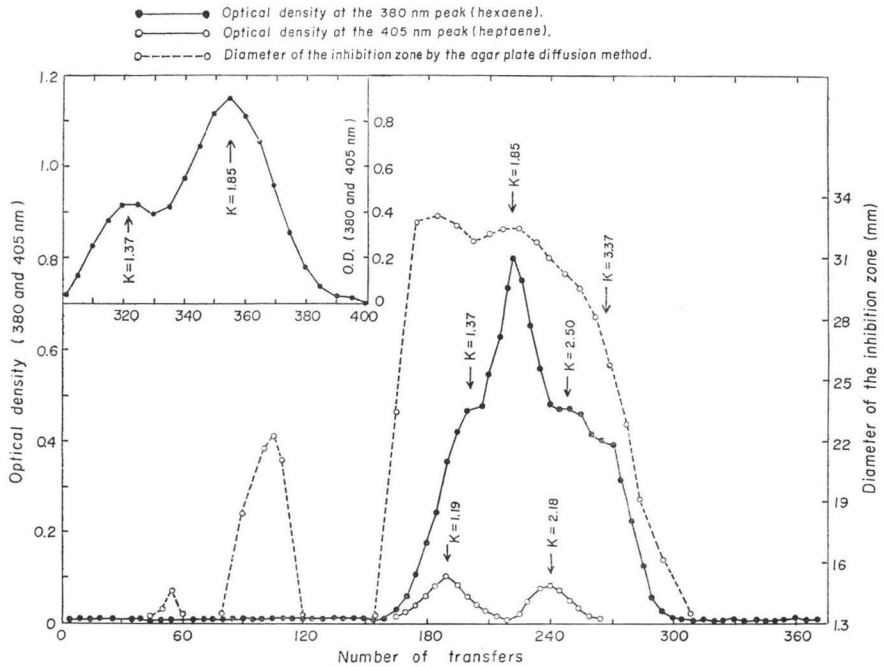
v/v/v) was about 25~30% higher than that obtained with butanol.

Between 50 and 80% of the candihexin was always found in the supernatant during the fermentation. In shake flasks fermentations, the proportion of antibiotic in the supernatant was smaller (45~60%) than in highly agitated fermentors (66~80%). This distribution was reversed by acidification of the broth prior to centrifugation (Table 2). About 63% of the total candihexin was found to be mycelium-associated after acidification of the broth to pH 5. An amount double that usually present in the mycelium was thus recovered from the mycelial cake. Based on these results, a procedure was outlined for pilot plant scale extractions of several candihexin batches. Preacidified culture broths were centrifuged and the mycelial cake and supernatant broth were extracted separately with chloroform-methanol (1:1, v/v) and with butanol respectively. The crude fractions obtained accounted for 69% of the total candihexin in the broth. Crude preparations of candihexin obtained in this way had $E^{1\%}_{1cm}$ values of 407, 468 and 560 at 379 nm.

Counter-current Distribution

In the solvent system I (pH 8.3) the main candihexin component had a K value of 3.36. Due to this high K value, resolution of the components was poor. The modified solvent system II (pH 6.25) gave better separation (Fig. 1). Four hexaene overlapping peaks were found in this solvent system after 350 transfers. They exhibited K values of 1.37, 1.85, 2.50 and 3.37. Two other heptaene peaks were found with K values of 1.19 and 2.18. Removal after 350 transfers of the polyene corresponding to peaks other than the main one (K=1.85, tubes

Fig. 1. Countercurrent distribution of the candihexin complex.
Insert: separation of the main component (K=1.85) after 550 transfers when the minor components (tubes 0~200 and 235~350) were removed after 350 transfers, and replaced by fresh solvent system II.



200~235) facilitated separation of the main component after 550 transfers (Fig. 1).

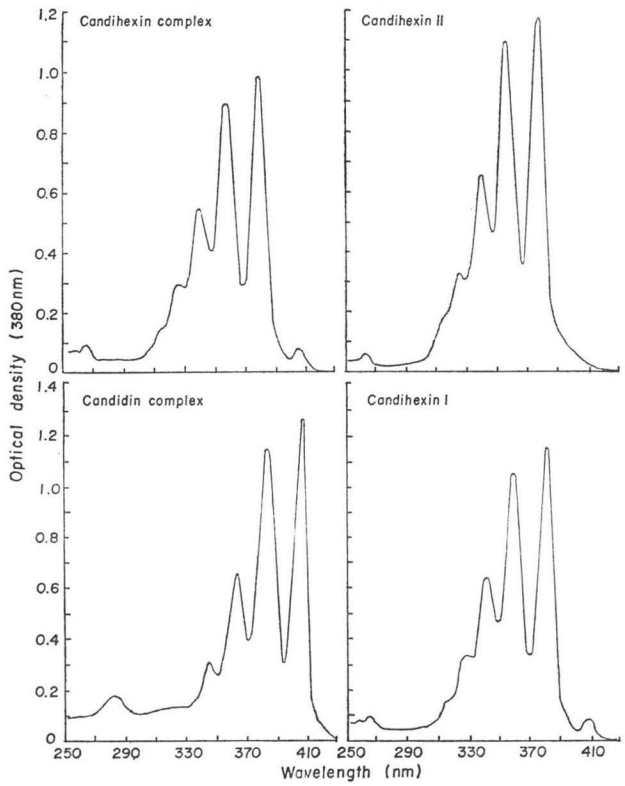
Bioassays of the CCD fractions indicated that almost all the antifungal activity was located in polyene-containing tubes (Fig. 1). Two other small inhibition peaks were found in fractions without any polyene activity (tubes 50~60 and 80~120) which suggest the co-production of small amounts of other non-polyene antifungal agents.

Purification and Properties of the Candihexin Complex

Candihexin crudes were purified in two steps by solvent fractionation (SF) in methanolic calcium chloride and partition CC on silicic acid. Upgrading of candihexin was accomplished by extraction of the crude candihexin with 3 % methanolic calcium chloride due to the selective formation of a complex with calcium chloride in methanol. Calcium chloride was removed by precipitation of the candihexin in water and by repeated washings of the water-saturated butanol solution of candihexin. Crops of 77~80 % pure candihexin were obtained from the ice-cooled concentrated butanol solution. Final removal of contaminants having similar solubility to candihexin was achieved by silicic acid partition CC. Highly colored impurities were eluted with the solvent front. Candihexin-containing pale yellow fractions without absorption in the 250~300 nm UV region were extremely pure. A crop of clusters of finely divided pale yellow needles of pure candihexin having an $E^{1\%}_{1cm}$ value of 1040 at 379 nm was obtained.

The pure candihexin complex was almost insoluble in water at neutral pH. Solubility increased in 0.01 N NaOH or HCl suggesting an amphoteric character. It is also insoluble in

Fig. 2. UV spectra of the candihexin complex, the candidin complex, candihexin I and candihexin II (10 μg/ml in methanol)



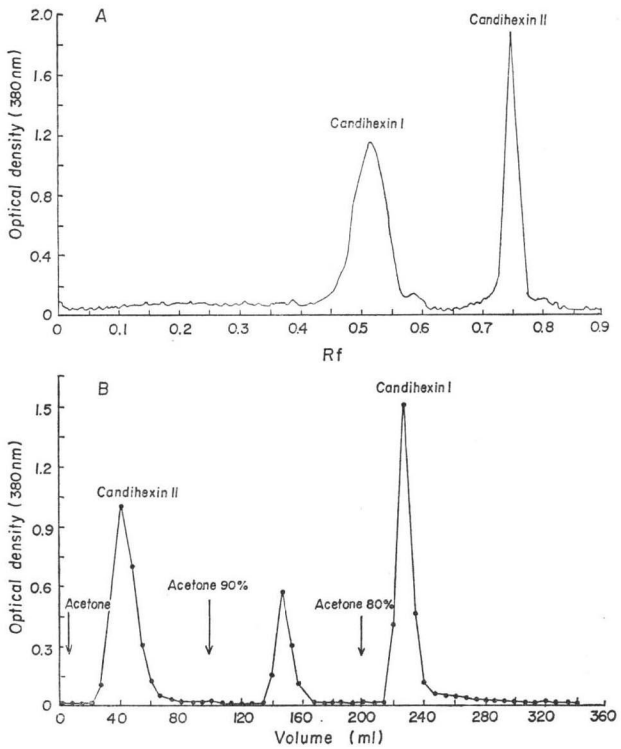
ether, benzene, slightly soluble in dry acetone, butanol and ethanol and more soluble in methanol. Solubility in lower alcohols and acetone increased considerably upon addition of 10~20% water. It was very soluble in water-saturated butanol, upper and lower phases of chloroform-methanol-water system, pyridine, glacial acetic acid, dimethyl formamide and dimethyl sulfoxide.

The UV spectrum in methanol showed a characteristic polyene spectrum with maxima at 379, 357, 340 and 323 nm (Fig. 2). In addition, it showed a small peak at 405 nm which was due to the presence in the complex of the two minor heptaene components detected by CCD. The sharp fine structure of the spectrum with a similar peak-spacing to that of the α, ω-disubstituted all-trans hexaenes[8] and the highest peak at the largest wavelength indicated a disubstituted all-trans chromophore.

Separations of Candihexins I and II

Two spots were identified by TLC in solvent system III with Rf values of 0.52 and 0.75 (Fig. 3A). These two fractions, designated as candihexins I and II, were also separated by

Fig. 3. A. Spectrodensitometric recording of the separation of candihexin I and II in Silicagel G TLC, using solvent system III.
B. Separation of candihexin I and II by partition CC on silicic acid using acetone-water mixtures as eluants



partition CC on silicic acid after elution with a discontinuous gradient of acetone-water mixtures of increasing polarity (Fig. 3B). Candihexin II (acetone-soluble) was eluted from the column with dry acetone. A small amount of degraded (abnormal UV spectrum) material was eluted with 90% acetone. Candihexin I (acetone-insoluble) remained adsorbed to the column, requiring

80% acetone to be eluted. Since candihexins I and II differ in acetone solubility they were also separated by dropwise addition of a methanol solution of candihexin in dry acetone while stirring. Candihexin I precipitates as pale-yellow needles while candihexin II remained in solution with a golden-yellow color. Two consecutive precipitations were required for a complete separation. Pure candihexin I and II had $E^{1\%}_{1cm}$ values of 986 and 1255, respectively.

Both candihexins I and II had a characteristic UV spectrum of hexaenes with maxima at 379, 357, 340 and 323 nm, but only candihexin I showed the peak at 405 nm (Fig. 2) indicating that the heptaenic minor components were absent from candihexin II.

Antifungal Activity of Candihexins I and II

Bioautography of candihexin fractions indicated one single inhibition zone with Rf 0.50 corresponding to candihexin I but no growth inhibition was produced by candihexin II. The MIC's of the candihexin complex and candihexins I and II for yeasts, filamentous fungi and

Table 3. Minimal inhibitory concentrations of candihexin complex and candihexins I and II (μg/ml)*

Test organisms	Candihexin complex	Candihexin I	Candihexin II
Yeasts and yeast-like fungi			
Candida lipolytica PP 47**	> 10	> 10	>100
Saccharomyces cerevisiae ATCC 9763	3	1	>100
Rhodotorula sp. PP 41	3	1	>100
Candida utilis NRRL Y900	3	1	>100
Candida albicans ATCC 18527	3	1	>100
Hansenula anomala PP 20	3	1	>100
Hansenula holstii NRRL Y2448	1	0.6	>100
Fungi			
Penicillium terrestre PP 84	6	3	>100
Penicillium chrysogenum ATCC 12690	6	3	60
Aspergillus niger ATCC 10254	6	3	>100
Aspergillus wentii PP 06	6	3	>100
Aspergillus sulfureus PP 17	10	6	>100
Trichoderma viride PP 24	10	6	>100
Mucor rouxii IMRU 80***	6	3	60
Bacteria			
Serratia marcescens IMRU 70	>100	>100	>100
Mycobacterium smegmatis IMRU 24	100	>100	100
Mycobacterium rhodochrous IMRU 21	60	>100	60
Sarcina lutea IMRU 14	30	>100	100
Bacillus cereus ATCC 12480	30	>100	60
Bacillus subtilis ATCC 7972	30	>100	60
Bacillus megaterium IMRU 10	30	>100	60
Escherichia coli B PP 01	>100	>100	>100
Staphylococcus aureus ATCC 6538P	30	>100	30

* Duplicate assays were made at the antibiotic dilutions indicated in methods. Results are average values.

** PP. Pilot plant culture collection.

*** IMRU. Institute of Microbiology, Rutgers University culture collection.

gram-positive and gram-negative bacteria are indicated in Table 3. The candihexin complex was active at the level of 3 μg/ml against all the yeast tested except *Candida lipolytica*. Filamentous fungi were inhibited by 6~10 μg/ml of candihexin. Some antibacterial activity was also found in crude preparations of the candihexin complex which was absent from the purified product. Candihexin I was slightly more active than the candihexin complex with MIC of 1 μg/ml against most yeasts and 3~6 μg/ml against filamentous fungi. Candihexin II showed no antifungal activity against either yeasts or filamentous fungi.

Discussion

Candihexin extraction from the culture broth, supernatant, or mycelial solids can be accomplished by addition of *n*-butanol or chloroform-methanol to make a diphasic system, the candihexin being extracted into the upper phase. A solvent/broth ratio of 1:1 (v/v) was found most convenient since lower recoveries of candihexin were obtained using low solvent/broth ratios, due to the unfavorable effect of the different ratio of volumes of the two phases formed on the partition constant K. The high amount of candihexin extracted with the solvent mixture chloroform-methanol, makes this solvent system the best choice for candihexin extraction. The change in the distribution of the antibiotic by flocculation of the microparticulate fraction of polyene after acidification, makes it possible to recover most of the candihexin in the batch from the mycelial cake. Total recovery of candihexin was higher than the values reported in the literature for other polyenes[9,10].

CCD of the candihexin complex in solvent system I used previously for separation of the heptaenes mycoheptin, levorin[11] and heptafungin[12] gives poor resolution of the candihexin components. The modified solvent system II provided good separation of the main candihexin components after 550 transfers. It indicated the presence of four main hexaene components, which was confirmed by further studies (MARTIN and MCDANIEL, in preparation). Solvent fractionation in methanolic calcium chloride[13] proved to be a good method for candihexin upgrading. Complete removal of the salt was required for further separation of the components. Partition CC gave the best results for final candihexin purification.

The characteristic UV spectrum of candihexin places this antibiotic in the hexaene group of polyene macrolides (2.2.4.1 of the BERDY classification)[14]. Pure candihexin showed no absorption in the 280~300 nm region as has been found with most other hexaenes. Absorption in this region is believed to indicate the presence of a tetraenic or trienoic ester chromophore.[8] Mediocidin (OWENS, Ph. D. Thesis, Rutgers University, 1972), tetraesin[15] and flavacid[16] are reported to have both a hexaene and a tetraene chromophore in the same molecule while endomycin (syn. helixin)[17] and plumbomycin[18] have been described as mixtures of tetraene and hexaene components. Cryptocidin, mycelin and hexin also possess a strong absorption in the 280~300 nm region although the presence of a tetraene chromophore has not been proved. None of the hexaene complexes so far described has an absorption peak at 405 nm or is coproduced with heptaenes. The heptaene components of the candihexin complex are identical with candidin, which was the only product of the parent strain (MARTIN and MCDANIEL, in preparation).

The candihexin complex and candihexin I have similar or higher antifungal activity than the values reported for other hexaenes but lower than those of the heptaenes. TLC and CC resolution of the candihexin complex into candihexins I and II suggests that candihexin like most other polyene antibiotics is a mixture of closely related components[19]. The different acetone solubility and antifungal activity of candihexins I and II reflect differences in the functional groups existing in the macrolide ring. Higher solubility of candihexin II in acetone indicates a less polar character, lacking or having substituted amino or hydroxy groups. The antifungal inactivity of candihexin II indicates the absence of bond formation between the polyene

macrolide and the sterol receptor site of the fungal cell membrane. Such lack of interaction can not be due to differences in the hydrophobic binding of the chromophore of candihexins I and II to the planar rings of the sterol molecule since both candihexins show identical all-trans hexaene spectra. More probably, it reflects differences in the binding of polar functions (amino or hydroxyl) to the 3-β-hydroxyl groups of the sterol molecule[20].

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