METABOLIC PRODUCTS OF MICROORGANISMS. 261[†] OBSCUROLIDES, A NOVEL CLASS OF PHOSPHODIESTERASE INHIBITORS FROM STREPTOMYCES

I. PRODUCTION, ISOLATION, STRUCTURAL ELUCIDATION AND BIOLOGICAL ACTIVITY OF OBSCUROLIDES A_1 TO A_4

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(Received for publication December 24, 1991)

A novel class of butyrolactones, named obscurolides, was isolated from the culture filtrate of *Streptomyces viridochromogenes* by chemical screening methods. The structural elucidation of the obscurolides A_1 to A_4 (**1**~**4**) is described. The carboxy group of the 4-aminobenzoic acid moiety of obscurolide A_1 (**1**) is reduced in the other compounds. The isolated natural products have been proved to be diastereomeric mixtures by a partial racemization at C-7 which belongs to an allylic alcohol system. The obscurolides showed a weak inhibitory activity against calcium/calmodulin-dependent and independent phosphodiesterases from bovine.

Chemical screening is an efficient method for detecting secondary metabolites of microorganism with different types of structures and biological activities[2~4]. In the course of our program we have discovered the obscurolides, excreted into the culture broth of *Streptomyces viridochromogenes* (strain Tü 2580), due to the remarkable yellow color on silica gel TLC plates, which is obtained after staining with EHRLICH's reagent. The purified compounds, named obscurolides, turned out to be inhibitors of cyclic AMP phosphodiesterase. In this paper we describe the isolation, characterization and structural elucidation of the obscurolides A_1 to A_4, which were identified as novel butyrolactone derivatives[5,6]. The following paper is engaged in the description of further minor compounds of the natural obscurolide complex[7].

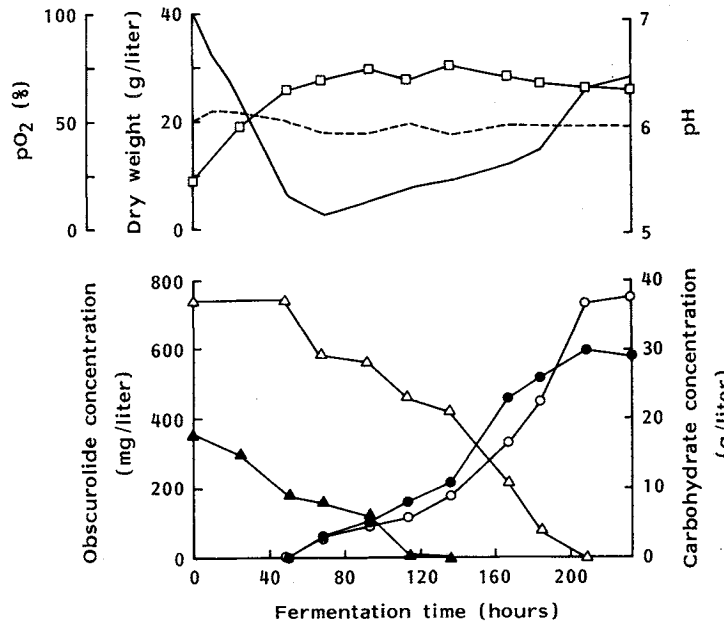
Fermentation and Isolation

Fermentation of *Streptomyces viridochromogenes* Tü 2580 was carried out in a medium containing

[†] See ref 1.

Fig. 1. Batch-fermentation of *Streptomyces viridochromogenes* (strain Tü 2580).

○—○ Obscurolide A_2 (2), ●—● obscurolide A_3 (3), ▽—▽ mannitol, ▼—▼ sucrose, □—□ dry weight, ------ pH, —— pO_2 (partial pressure of oxygen).



sucrose 2%, mannitol 4% and soybean meal 2%. The time course of the production of the obscurolides in a 10-liter jar fermenter is shown in Fig. 1. Secondary metabolite production started at 48 hours after incubation, and maximum accumulation was observed after a 216-hour incubation period at which the concentrations of the obscurolides were A_2 760 mg/liter and A_3 600 mg/liter. The production was strongly dependent on pH value of the culture broth and maximal concentrations were obtained when the pH was maintained at pH 6 during the whole fermentation cycle.

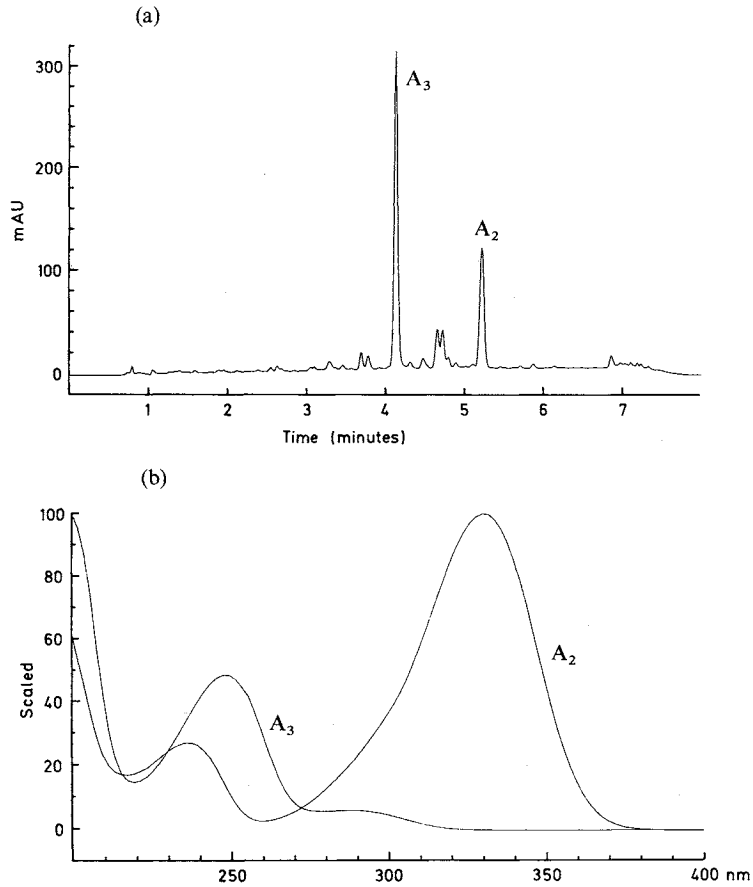
Production of the obscurolides was determined by HPLC on reversed-phase silica gel by direct injection of the culture filtrate. Minor components of the obscurolide complex were identified by photodiode array detection comparing the UV/Vis spectra of all peaks which were monitored during the HPLC run with those of the main compounds[8]. An elution profile of the culture filtrate and UV spectra of the main compounds A_2 and A_3 is shown in Fig. 2.

Isolation of the obscurolides was carried out by adsorption of the culture filtrate on Amberlite XAD-16, desorption with 70% acetone and extraction of the concentrate with ethyl acetate. The raw product was purified by repeated flash chromatography on silica gel with $CHCl_3$ - MeOH mixtures. Pure obscurolides A_1 to A_4 were obtained after chromatography on Sephadex LH-20 with methanol in a ratio of approximately 0.1 : 1 : 1 : 0.1.

Characterization

The obscurolides were obtained as pale yellow, amorphous powders (A_1, A_2) or as oily residues (A_3, A_4), which were easily soluble in acetone, methanol or dimethyl sulfoxide, hardly soluble in ethyl acetate or chloroform and insoluble in water or alkanes. They gave characteristic coloration reactions with EHRLICH's reagent (yellow) and vanillin-sulfuric acid (brown). Their Rf values (TLC) and retention times

Fig. 2. HPLC elution profile of culture filtrate of *Streptomyces viridochromogenes* (strain Tü 2580) plotted at 240 nm (a), and UV spectra of obscurolides A_2 and A_3 recorded during the HPLC run (b).



(HPLC) are given in Table 1.

The molecular formulae of the obscurolides could be obtained by high resolution mass spectrometry (HR-MS) showing a skeleton of fifteen carbon atoms with small variations among the components. The IR spectra (Fig. 3, Table 1) displayed complex patterns of absorption bands with a recurrent band near 1770 cm^{-1} indicating a butyrolactone moiety. An additional carbonyl group was observed for obscurolide A_1 and A_2. This could explain the differences in the UV spectra (methanol), which were typical for substituted benzene derivatives. The ^1H and ^{13}C NMR spectra (Tables 2 and 3) confirmed that the obscurolides A_1 to A_4 are closely related. Remarkable are the signals of a *para*-substituted benzene and of an aliphatic moiety, in which adjacent protons gave a well interpretable coupling pattern. The differences between the four components could be traced back to one functional group, which is attached to the benzene ring. Some of the NMR signals are split due to isomers in the ratio of 3:2. Efforts to separate the isomers by chromatographical methods failed. Initially this fact seemed to be very obscure and led to the name of these secondary metabolites.

Structure of Obscurolide A_3 (3)

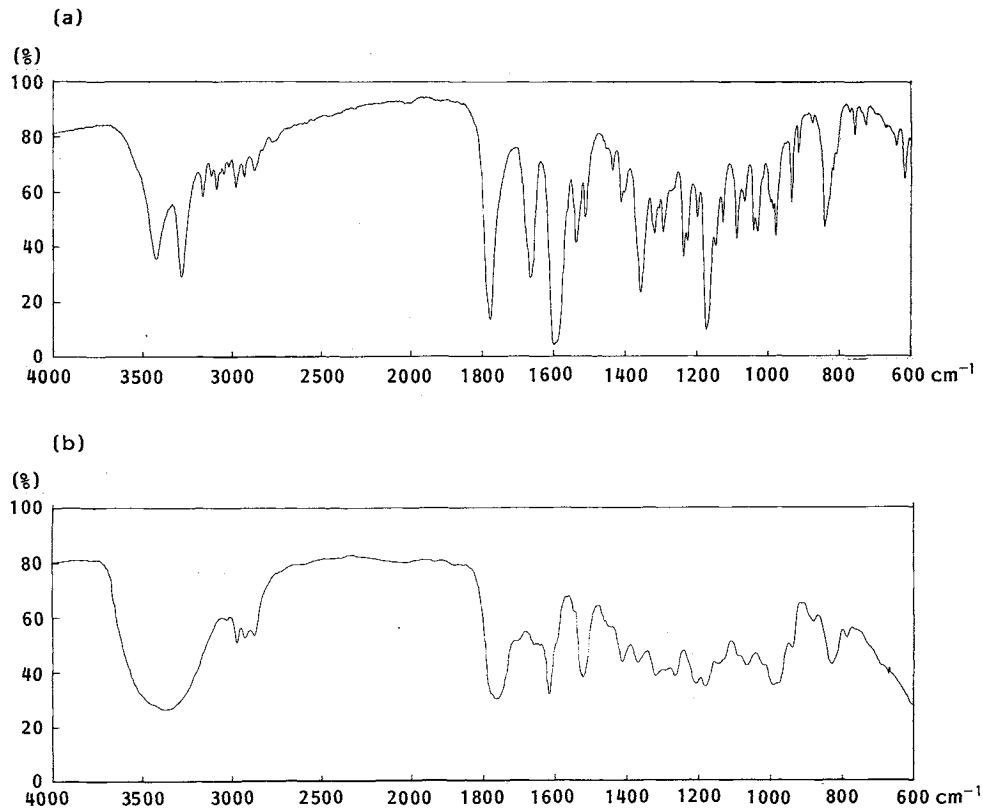
In the ^1H NMR spectrum (Table 2) of obscurolide A_3 ($C_{15}H_{19}NO_4$), one of the main components, all nineteen hydrogen atoms are observable, three of them were exchangeable by D_2O indicating hydroxy

Table 1. Characterization of the obscurolides A_1 to A_4.

	A_1 (1)	A_2 (2)	A_3 (3)	A_4 (4)
Molecular formula	$C_{15}H_{17}NO_5$	$C_{15}H_{17}NO_4$	$C_{15}H_{19}NO_4$	$C_{16}H_{21}NO_4$
HREI-MS (M^+)	291.1098	275.1168	277.1319	291.1475
(Calcd value)	(291.1108)	(275.1159)	(277.1315)	(291.1472)
IR (KBr) cm^{-1}	3380, 1765, 1680	3420, 1775, 1665	3470, 1760, 1615	3460, 1770, 1615
Rf value[a] I	0.33	0.35	0.21	0.56
II	0.13	0.13	0.06	0.23
Retention time (minutes)[b]	4.70	5.18	4.11	5.90

[a] TLC silica gel, I: $CHCl_3$-MeOH (9:1), II: Petroleum ether-EtOAc (1:1).
[b] HPLC, see Experimental.

Fig. 3. IR spectra of obscurolides A_2 (2) (KBr, a) and obscurolide A_3 (3) (film, b).



groups (δ 3.86 t, $J=6.0$ Hz; δ 3.92 d, $J=4.5$ Hz) and a NH group (δ 5.50 d, $J=7.0$ Hz). The *para*-substituted benzene gave two doublets (δ 6.64 and 7.15, $J=8.5$ Hz), each signal for two protons. In the ^{13}C NMR spectrum (Table 3) besides six benzene carbon atoms there could be seen one olefinic double bond (δ 125.4, 140.0), three methine groups attached to heteroatoms (δ 55.9, 67.3, 85.5), two methylene groups (δ 35.4, 64.6), one methyl group (δ 23.7) and one carbonyl group (δ 175.6). The connectivity of the aliphatic and olefinic moieties was proved by a ^1H-^1H COSY spectrum (Fig. 4). Starting from the methyl group (δ 1.21 d, $J=7.0$ Hz) the chain could be derived as $-CH_2-{}^3CH-{}^4CH-CH=CH-{}^7CH-CH_3$. Taking into

Table 2. ^{1}H NMR signals in acetone-d_6 (δ in ppm relative to internal TMS) of the obscurolides A_1 to A_4 (**1**~**4**) and the derivatives A_2-monoacetate (**6**) and dehydro-A_2 (**8**).

Proton	Obscurolides				Derivatives	
	A_1 (**1**)	A_2 (**2**)	A_3 (**3**)	A_4 (**4**)	**6**[a]	**8**
2-H_a	2.50 dd	2.54 dd	2.42 dd	2.43 dd	2.52* dd	2.59 dd
2-H_b	3.12 dd	3.15 dd	3.07 dd	3.08 dd	3.06* dd	3.19 dd
3-H	4.20~4.39 m	4.20~4.33 m	4.13 m	4.13 m	4.16 m	4.52 m
3-NH	6.24 br d	6.54 br d	5.50 br d	5.51 br d	4.78~4.90 m	6.62 br d
4-H	4.82 m	4.90 m	4.82 m	4.83 m	4.78~4.90 m	5.12 ddd
5-H/6-H	5.76~6.06 m	5.85~6.06 m	5.80~6.00 m	5.78~6.01 m	5.79 dd/5.90 dd	7.04 dd/6.31 dd
7-H	4.20~4.39 m	4.20~4.40 m	4.32 m	4.31 m	5.38 m	—
7-OH	3.97 d	3.99 d	3.92 d	3.96 d	—	—
8-CH_3	1.20* d	1.22* d	1.21* d	1.21* d	1.33 d	2.27 s
2'-H/6'-H	6.72 d	6.80 d	6.64 d	6.63 d	6.62 d	6.82 d
3'-H/5'-H	7.83 d	7.70 d	7.15 d	7.10 d	7.73 d	7.69 d
7'-H	—	9.74 s (1H)	4.48* d (2H)	4.26 s (2H)	9.76 s (2H)	9.74 s (1H)
7'-OH	—	—	3.86 t	—	—	—
CH_3O	—	—	—	3.24 s	—	—

[a] For further signals see Experimental.
* Split signal, main peak.

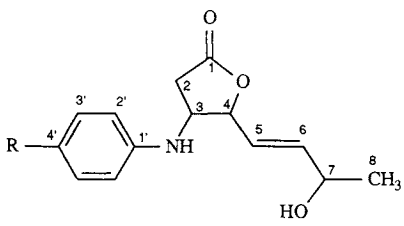
Table 3. ^{13}C NMR signals of the obscurolides A_1 to A_4 (**1**~**4**) and their derivatives **5** to **8**.

Carbon No.	Multiplicity[a]	Obscurolides				Derivatives			
		A_1 (**1**)	A_2 (**2**)	A_3 (**3**)	A_4 (**4**)	**5**[b]	**6**[b]	**7**[b]	**8**
1	s	175.1	175.0*	175.6	175.5	175.2	174.7	174.3*	174.7
2	t	35.3	35.3	35.4	35.5	35.3	35.2*	34.4*	34.9*
3	d	55.5*	55.4*	55.9*	55.9	55.8	55.3*	54.3*	54.8*
4	d	85.4	85.3*	85.5*	85.5*	85.1	84.9	83.9*	83.6
5	d	125.1*	125.0*	125.4*	125.4*	128.3*	128.0	124.8	141.5
6	d	140.6	140.8	140.0	140.2	134.5	135.0	134.1*	132.0
7	d	67.3*	67.3*	67.3*	67.4*	70.1	70.0	68.9*	197.6[c]
8	q	23.6	23.7	23.7	23.7	21.1	21.1	20.1*	27.5
1'	s	151.9*	153.3	147.0	147.5*	148.0	153.3*	150.6*	153.0
2'/6'	d	112.8	113.3	113.9	113.7*	113.8	113.3*	112.7	113.4*
3'/5'	d	132.4	132.7	129.1	130.2	131.0	132.6	132.3*	132.7
4'	s	119.9	128.0	132.4	128.3	126.2	128.1	127.4*	128.3
7'	s	174.1	190.3	64.6	74.9	66.7	190.3	190.3	190.3
8'	q	—	—	—	57.5	—	—	—	—

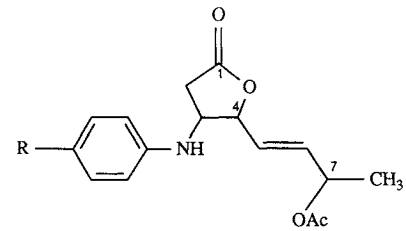
[a] From attached proton transfer spectra.
[b] For further signals see Experimental.
[c] Singlet.
* Split signal, main peak.

account the chemical shifts of the methine protons, C-3 should be attached to NH and C-4 as well as C-7 to oxygen. 2-H_a and 2-H_b (δ 2.42, 3.07, $J_{2a,2b}=17.5$ Hz, $J_{2a,3}=4.5$ Hz, $J_{2b,3}=8.0$ Hz) should be neighboring the carbonyl group within a butyrolactone ring. The second methylene group gave cross peaks to a hydroxyl group (δ 3.86) and one pair of the aromatic protons (δ 7.15, $J<0.5$ Hz) indicating a hydroxymethylene group at the benzene ring. The connection between the aliphatic chain and the benzene ring is effected by the NH-group at C-3. Taken together these data led to the constitution **3** of obscurolide A_3 in accordance with the fragmentation pattern of the molecule in the EI-mass spectrum. The favored fragmentation

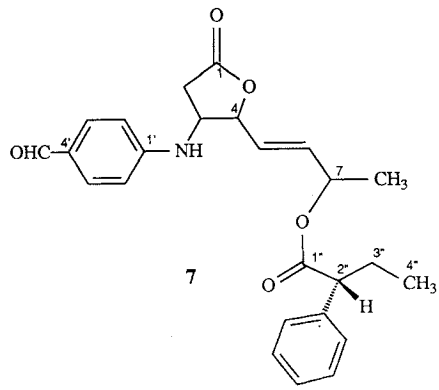
Scheme 1. Structures of the obscurolides and their derivatives.



1 R=COOH
2 R=CHO
3 R=CH_2OH
4 R=CH_2OCH_3



5 R=CH_2OAc
6 R=CHO



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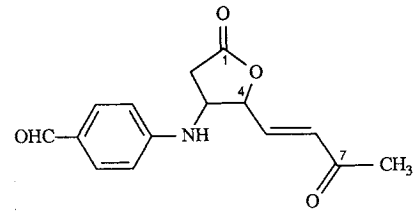

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takes place within the butyrolactone ring by the loss of $C_5H_9O_2$ (101) or $C_6H_8O_3$ (128) leading to fragment ions at *m/z* 176 and 149, respectively. The complete assignment of the ^{13}C NMR signals was done by a HETCOR NMR experiment. The *E*-configuration of the allylic double bond was derived from the coupling constant (5-H/6-H, $J=15.5$ Hz) of obscurolide A_3-diacetate (**5**).

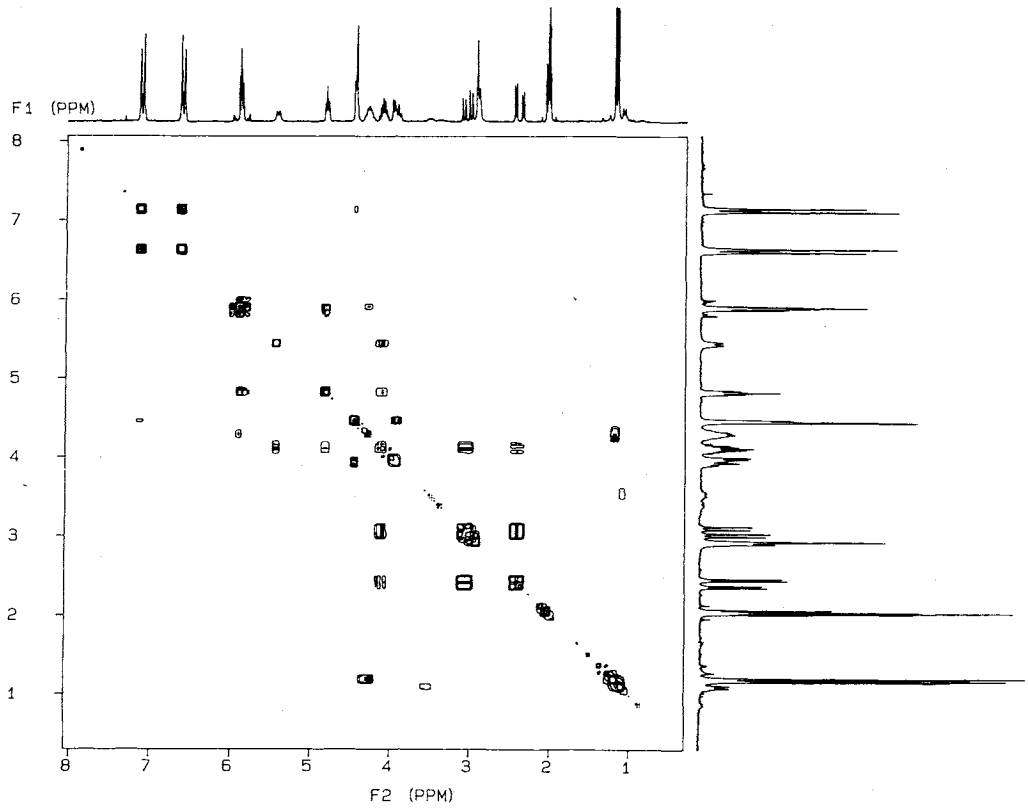
Structure and Derivatives of Obscurolide A_2 (**2**)

The second main component, obscurolide A_2 ($C_{15}H_{17}NO_4$), differed from the component A_3 (**3**) by two hydrogen atoms. The dehydration from A_3 to A_2 resulted in an additional carbonyl absorption band (1665 cm^{-1}) in the IR spectrum (Fig. 3). The ^1H and ^{13}C NMR spectra are closely related to those of **3** with the exception that instead of the hydroxymethylene group of **3** an aromatic aldehyde group ($\delta_H=9.74$, $\delta_C=190.3$) is present. The full assignment of the NMR data (Tables 2 and 3) of obscurolide A_2 (**2**) was done in analogy to **3** and in accordance with substituent calculation for the *para*-substituted benzene ring. During the acetylation reaction the weak basic NH group again remained unchanged, the monoacetate **6** was isolated.

Structure of Obscurolide A_1 (**1**) and A_4 (**4**)

The molecular formula of obscurolide A_1 ($C_{15}H_{17}NO_5$) showed an additional oxygen compared with **2**. In the ^1H and ^{13}C NMR spectra the signal of the aldehyde disappeared and an additional signal appeared at $\delta_C=174.1$ typical for a carboxylic acid. From the whole data the structure of this compound

Fig. 4. ^1H-^1H COSY NMR spectrum of obscurolide A_3 (**3**) in acetone-d_6 at 200 MHz.



was assigned as **1**.

The second minor compound of the A-series, obscurolide A_4 ($C_{16}H_{21}NO_4$), could be assigned as *O*-methyl ether (**4**) of **3**. The ^1H and ^{13}C NMR spectra led to an additional methoxy group (δ_H 3.24, δ_C 57.5). The other signals (Tables 2 and 3) were very similar to those of **3** with the exception of the lack of the 7′-OH triplet.

Stereochemical Characteristic

The pure obscurolides showed doublings of some NMR signals, which indicated diastereomers in the ratio 3:2. In the ^1H NMR spectrum of obscurolide A_2 (**2**) for instance, the signal of the terminal methyl group (8-H_3: δ 1.220 and 1.215) was split slightly but significantly. In the ^{13}C NMR spectra the same effect was observable for the signals of C-1, C-3, C-4, C-5 and C-7, with a maximum distance of the signals belonging together of 0.23 ppm. This data suggested that the reason for the disturbance lay in the allylic side chain. A change of the *cis*/*trans* position of the substituents at the butyrolactone ring or an isomerization of the double bond was excluded due to the small differences in the chemical shifts and the unchanged coupling constants.

The determination of the configuration of the secondary alcohol at C-7 was carried out according to the method of HELMCHEN[9]. The product of the conversion of **2** with (−)-(*R*)-2-phenylbutyric acid is the phenylbutyrate **7**, whose molecular formula resulted from the mass spectrum (HR-MS). The constitution was derived from the NMR data, especially due to the fact, that the signal of 7-H was

shifted 1.1 ppm downfield. The occurence of double signals (in a 3 : 2 ratio) in the NMR spectra was now more significant, particularly in the area of the phenylbutyryl moiety. In the case of the main component the signals of the protons "left" to C-7 (2-H_2 to 6-H) were shifted to higher field ($\Delta\delta$ 0.06 to 0.33 ppm) compared with the minor component and the acetate **6** (Table 4). In this case C-7 has (*R*)-configuration. Out of the signals of the minor component only 8-H_3 was shifted to lower field, thus C-7 has (*S*)-configuration. The conclusion of this data is, that obscurolide A_2 (**2**) exists in two diastereomers at C-7 with a preponderance of the (7*R*)-diastereomer. We suppose the same reason for the double signals of the other obscurolides. There was no evidence for a racemization of this center of chirality during the work-up procedure, because a fermentation at pH 7 and a work-up without acid let to diastereomers in the same ratio.

Table 4. ^{1}H NMR signals (δ in ppm relative to internal TMS) of 7-*O*-acetyl-obscurolide A_2 (**6**) and of the diastereomers (**7a**/**7b**)[a] of 7-*O*-[(*R*)-2-phenylbutyryl]-obscurolide A_2 (**7**) in acetone-d_6.

Proton	**6**	**7a**[b]	**7b**
2-H_a	2.56	2.48	2.55
2-H_b	3.14	3.00	3.07
3-H	4.38	4.10	4.31
4-H	4.90	4.78	4.88
5-H	5.98	5.61	5.94
6-H	5.98	5.86	5.96
7-H	5.37	5.40	5.40
8-H_3	1.28	1.29	1.20
2′-H/6′-H	6.80	6.72	6.78

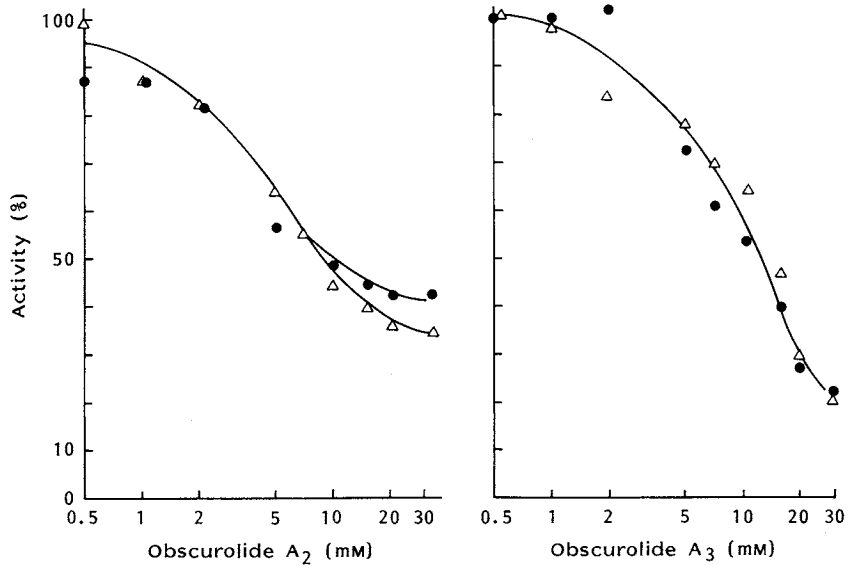
[a] Data from the mixture.
[b] Main component.

Furthermore **2** was converted into the dehydroobscurolide A_2 (**8**, $C_{15}H_{15}NO_4$) by gentle oxidation with manganese(IV)-oxide in acetone[10]. The oxidation took place at C-7 of **2**. This could be deduced from the ^{1}H NMR spectrum (Table 2), which showed a downfield shift of 8-H_3 (δ_H from 1.22 to 2.27) accompanied by a change into a singlet. The olefinic protons 5-H/6-H were much more separated ($\Delta\delta$ 0.73 ppm, $J_{5,6}=16.1$ Hz). The lack of the coupling constants proceeding of 7-H in the obscurolides allowed the interpretation of the coupling pattern of 4-H within **8**. According to the size of $J_{3,4}=4.1$ Hz the substituents at the butyrolactone ring should have *trans* configuration. The other values of the allylic system are $J_{4,5}=5.5$ Hz and $J_{4,6}=1.5$ Hz. Conspicuous in the ^{13}C NMR spectrum was the lack of a methine group signal and the appearance of a new carbonyl group signal at δ_C 197.6. In contrast to the ^{1}H NMR spectrum with its sharp signals, the ^{13}C NMR spectrum in DMSO-d_6 at 35°C showed double signals, which collapsed by heating to 100°C and separated again by cooling. This indicated two distinguishable conformers at lower temperatures. Thus, there exists two reasons for the double signals of the obscurolides: i) The partial racemization at C-7 and ii) conformational effects of the molecular framework, *e.g.* caused by the rotation around the NH-group bonds.

Biological Properties

The obscurolides were tested as inhibitors to two cAMP phosphodiesterase isozymes. The calcium/calmodulin-dependent phosphodiesterase from bovine brain was inhibited by the aldehyde (A_2) and somewhat less by the alcohol (A_3). IC_{50} values were 8 and 12 mmol/liter (Fig. 5). When the same compounds were tested with the calcium-independent enzyme from bovine heart, almost identical concentrations were required for half maximal inactivitation (Fig. 5). Due to solubility problems in the assay, concentrations which would completely inhibit enzyme activity, could not be tested with this isozyme. Obscurolides A_1 and A_4 exhibited the best potency (Table 5) indicating that further chemical modification may be meaningful for the development of compounds with higher activity. The growth inhibiting potency of the obscurolides against bacteria, yeast and fungi was tested. No effect was observed with concentrations up to 15 mmol/liter.

Fig. 5. Dose dependency of inhibition of cAMP hydrolysis by obscurolide A_2 (**2**) and A_3 (**3**) using calcium/calmodulin-dependent phosphodiesterase from bovine brain (●) and calcium-independent phosphodiesterase from bovine heart (△).



Discussion

The obscurolides are new 3,4-substituted butyrolactones which could be built up biogenetically from acetate (C-1 to C-8) and *p*-aminobenzoic acid (shikimic acid pathway). Therefore obscurolide A_1 represents the initial component of the A-series and could be reduced predominently to the aldehyde **2** and the alcohol **3**. The methyl ether **4** could be produced from **3** in the presence of *S*-adenosylmethionin. The various enzymatic activities of the strain are remarkable, which results in a modification of the basic framework recognizable by the large number of minor components[7]. It is worth mentioning that the natural products show a partial racemization at C-7. Due to the fact that the diastereomers occur even under gentle isolation conditions, we suppose that the reduction of a former carbonyl group in this position is not subject to a stereospecific control during the biosynthesis. The absolute configuration of C-3 and C-4 is not determined yet.

Table 5. Effect of the obscurolides on calcium/calmodulin-dependent phosphodiesterase from bovine brain.

Obscurolide	IC_{50} (mM)
A_1 (**1**)	15
A_2 (**2**)	8
A_3 (**3**)	12
A_4 (**4**)	2

Tested in a simple enzyme assay, the obscurolides inhibited calcium/calmodulin-dependent and independent phosphodiesterases from bovine. Compared with known specific phosphodiesterase inhibitors the activity of the obscurolides was weak. Essentially no discrimination among the isozymes was observed. The slightly better potency of the aldehyde compared to the alcohol may be due to formation of Schiff-bases with the amino groups of proteins. However, the activity of the alcohol indicates that this may at best be a minor effect. This is supported by the even better activity of obscurolide A_4, the methoxy methyl derivate. It is conceivable that further chemical modification of the obscurolides, *e.g.* at the 4'-position, may yield phosphodiesterase inhibitors with a higher potency. Since distinctly stereospecific inhibition of a calmodulin-independent phosphodiesterase was described for a substituted phenyl-2-pyrrolidone[11], it may also be meaningful to investigate a potential stereospecific activity of the obscurolides as phosphodiesterase inhibitors once the separation of the racemates into isomers has been accomplished.

Experimental

General

MP's were determined with a Reichert hot stage microscope. UV spectra were recorded using a

Kontron Uvikon 860 spectrometer. IR spectra obtained using a Perkin-Elmer Model 298 spectrometer. The NMR spectra (δ in ppm relative to internal TMS) were determined using a Varian VXR-200. The electron impact mass spectra (EI-MS, 70 eV) were obtained on a Varian MAT 731 and on a Finnigan MAT 311 A, using direct probe insert, high resolution with perfluorokerosine as a standard. A Perkin-Elmer Model 241 polarimeter was used for recording of the optical rotations.

Analytical

TLC was performed on silica gel plates (Machery-Nagel SIL G/UV 254+366, 0.25 mm silica gel on glass). Production of the obscurolides was determined by HPLC. A sample of the culture broth was centrifuged (10 minutes, 12,000 × *g*) and 10 μl of the supernatant were injected onto a Shandon ODS Hypersil column (5 μm) 125 × 4.6 mm, fitted with a precolumn 20 × 4.6 mm. The samples were separated by gradient elution with a flow rate of 2 ml/minute; solvent A was 0.1% phosphoric acid, solvent B was acetonitrile. The linear gradient was from 0 to 40% B in 6 minutes, increasing to 100% B in 1 minute with a 1-minute-hold at 100% B, followed by a period of 5 minutes under initial conditions. The chromatographic system consisted of a HP 1090M liquid chromatograph equipped with a diode array detection system and workstation (Hewlett-Packard). The compounds were detected by UV absorbance at 240 nm.

Production Organism

The producing strain Tü 2580 was a new soil isolate from Mayes Canyon (Peru) and was classified according HÜTTER and BERGEY as *Streptomyces viridochromogenes*.

Fermentation

Streptomyces viridochromogenes Tü 2580 was cultivated in a 10-liter fermenter (Biostat E, B. Braun-Diessel Biotech). 9.5 liters of production medium containing 2% sucrose, 4% mannitol, 2% degreased soybean meal, 0.01% $CaCl_2$, 0.01% KCl, 0.01% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 0.001% $CoCl_2$, 0.001% $CuSO_4 \cdot 7H_2O$ and 0.001% $ZnSO_4 \cdot 7H_2O$ in tap water pH 6.0 were inoculated with 0.5 liter of shaking cultures grown 48 hours in a medium consisting of mannitol 2% and soybean meal 2% in tap water (pH 7.5) at 27°C and 120 rpm. Fermentation was carried out for 10 days with agitation at 250 rpm and airing of 4 liters/minute at 27°C. The pH was maintained at pH 6 with 3 M H_2SO_4 and 3 M NaOH. The growth was measured by dry cell weight.

Phosphodiesterase Assay

Calmodulin-independent phosphodiesterase from bovine heart was purchased from Boehringer Mannheim. Calmodulin-dependent phosphodiesterase was isolated from bovine brain to apparent homogeneity by calmodulin-affinity chromatography[12]. Calmodulin was purified by phenylsepharose hydrophobic chromatography[13]. Phosphodiesterase activity was measured by the method of PÖCH[14]. The incubations contained 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM AMP, and 20 μmol/^{3}H-cAMP (20 nCi) as a substrate. Either 1 mM EGTA or 1 mM Ca^{2+} and 0.5 μg calmodulin were added to measure respective enzyme activities. The enzyme assay was linear with respect to protein concentration and incubation time, and activation of the respective isozyme by Ca^{2+} and calmodulin was maximal. Obscurolides were added to the incubations in the indicated final concentrations from a DMSO - water (1 : 4) stock solution. Controls with corresponding amounts of DMSO indicated no interference with the assay by the solvent. The total assay volume was 500 μl. IC_{50} values (concentrations required for 50% inhibition) were estimated graphically from respective plots. All assays were performed in duplicate and the means are presented (Fig. 5, Table 5).

Isolation

After adding of 2% Hyflo Super-cel the culture broth (10 liters) was filtered. The culture filtrate was passed through an Amberlite XAD-16 column (Rohm & Haas). Impurities were washed out with acetone - water (1 : 9) and the obscurolides were desorbed with acetone - water (7 : 3). After removing the organic solvent by evaporation *in vacuo*, the aqueous residue was extracted at pH 7 four times with neutralized ethyl acetate and the combined organic layers were again evaporated to dryness *in vacuo* to give 10 g raw material. 2.5 g of the material was purified by flash-chromatography (column 40 × 5 cm, silica gel 40 μm, $CHCl_3$ - MeOH, 9 : 1) yielding 1.9 g obscurolide-complex. This was applied on a further silica

gel column (40 × 3 cm, $CHCl_3$ - MeOH, 15 : 1) to give 0.8 g obscurolide A_3, 50 mg obscurolide A_4 and 0.8 g of a mixture of the obscurolides A_2 and A_1. These components were separated by gel-filtration on a Sephadex LH-20 column (100 × 2.5 cm, MeOH) yielding 0.7 g obscurolide A_2 and 60 mg A_1.

Obscurolide A_1 (**1**)

$[\alpha]_D^{20}$ +20° (*c* 0.2, MeOH); Rf values see Table 1; IR (film) see Table 1; UV λ_{max} (MeOH) nm (ε) 203 (14,600), 222 (8,000), 297 (20,300); ^{1}H NMR see Table 2; ^{13}C NMR see Table 3; EI-MS see Table 1.

Obscurolide A_2 (**2**)

MP 125°C; $[\alpha]_D^{20}$ +35° (*c* 0.3, MeOH); Rf values see Table 1; IR (KBr) see Fig. 3; UV λ_{max} (MeOH) nm (ε) 202 (14,100), 237 (6,700), 325 (23,900); ^{1}H NMR see Table 2; ^{13}C NMR see Table 3; EI-MS see Table 1.

Obscurolide A_3 (**3**)

$[\alpha]_D^{20}$ +45° (*c* 0.2, MeOH); Rf values see Table 1; IR (film) see Fig. 3; UV λ_{max} (MeOH) nm (ε) 205 (24,600), 253 (17,700), 295 (2,900); ^{1}H NMR see Table 2; ^{13}C NMR see Table 3; EI-MS see Table 1.

Obscurolide A_4 (**4**)

$[\alpha]_D^{20}$ +42° (*c* 0.2, MeOH); Rf values see Table 1; IR (film) see Table 1; UV λ_{max} (MeOH) nm (ε) 205 (16,200), 254 (11,600), 294 (1,300); ^{1}H NMR see Table 2; ^{13}C NMR see Table 3; EI-MS see Table 1.

Obscurolide A_3-diacetate (**5**)

Obscurolide A_3 (**3**, 27.7 mg) was dissolved in 0.5 ml CH_2Cl_2 and treated at 0°C with 24 ml acetic anhydride and 20 ml pyridine. After one hour stirring at ambient temperature, the reaction mixture was poured on 10 ml ice-water and extracted with CH_2Cl_2. The extracts were dried over Na_2SO_4, evaporated *in vacuo* and the crude product was chromatographed on silica gel (column 30 × 2 cm, ethyl acetate - petroleum ether, 1 : 1) yielding 11.2 mg (31%) **5**; Rf values 0.82 ($CHCl_3$ - MeOH, 9 : 1), 0.55 (ethyl acetate - petroleum ether, 1 : 1); IR (film) cm^{-1} 3500, 3370, 1780, 1725, 1615, 1525; UV λ_{max} (MeOH) nm (ε) 204 (22,900), 256 (15,900), 294 (1,200); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.31 (3H, d, 8-H_3), 2.06 (6H, s, acetyl-CH_3), 2.42* (1H, d, 2-H_a), 2.99* (1H, dd, 2-H_b), 3.90~4.10 (2H, m, 3-H, NH), 4.82 (1H, m, 4-H), 4.98 (2H, s, 4′-CH_2), 5.36 (1H, m, 7-H), 5.76 (1H, dd, 5-H), 5.87 (1H, dd, 6-H), 6.54 (2H, d, 2′-H, 6′-H), 7.20 (2H, d, 3′-H, 5′-H); ^{13}C NMR (50.3 MHz, acetone-d_6) see Table 3, δ 20.3, 20.9 (q, acetyl-CH_3), 170.2, 170.9 (s, acetyl-CO); EI-MS *m/z* 361 (M^+).

Obscurolide A_2-monoacetate (**6**)

27.5 mg obscurolide A_2 (**2**) were acetylated and purified as described before with 2.2 equivalents of acetic anhydride - pyridine. 16.9 mg (53%) **6** were obtained. Rf values 0.73 ($CHCl_3$ - MeOH, 9 : 1), 0.45 (ethyl acetate - petroleum ether, 1 : 1); IR (film) cm^{-1} 3500, 3350, 1780, 1725, 1670, 1600, 1530; UV λ_{max} (MeOH) nm (ε) 203 (13,400), 238 (6,700), 325 (25,100); ^{1}H NMR (200 MHz, $CDCl_3$) see Table 2, δ 2.07 (3H, s, acetyl-CH_3); ^{13}C NMR (50.3 MHz, acetone-d_6) see Table 3, δ 20.2 (q, acetyl-CH_3), 170.2 (s, acetyl-CO); EI-MS *m/z* 317 (M^+).

7-*O*-(2-Phenylbutyryl)-obscurolide A_2 (**7**)

27.5 mg obscurolide A_2 dissolved in 3 ml THF was mixed at 0°C with 18.0 mg (−)-(*R*)-2-phenylbutyric acid, 22.7 mg dicyclohexylcarbodiimide and a catalytic amount of DMAP. After stirring for one hour at ambient temperature, the mixture was extracted with 10% $NaHCO_3$ solution. The organic phase was dried over Na_2SO_4 and evaporated *in vacuo*. Chromatography on silica gel (column 20 × 2 cm, ethyl acetate - petroleum ether, 1 : 2) yielded 32.2 mg (76%) **7**. Rf values 0.80 ($CHCl_3$ - MeOH, 9 : 1), 0.58 (ethyl acetate - petroleum ether, 1 : 1); IR (film) cm^{-1} 3500, 3080, 1780, 1725, 1705, 1670, 1600; UV λ_{max} (MeOH) nm (ε) 204 (19,900), 235 (7,200), 325 (25,100); ^{1}H NMR (200 MHz, acetone-d_6) δ 0.87 (3H, t, 4″-H_3), 1.29 (3H, d, 8-H_3), 1.64~1.88 (2H, m, 3″-H_2), 2.48 (1H, dd, 2-H_a), 3.00 (1H, dd, 2-H_b), 3.54 (1H, t, 2″-H), 4.10 (1H, m, 3-H), 4.78 (1H, m, 4-H), 5.40 (1H, m, 7-H), 5.61 (1H, ddd, 5-H), 5.86 (1H, ddd, 6-H), 6.50 (1H, d, NH), 6.72 (2H, d, 2′-H, 6′-H), 7.32 (5H, m, C_6H_5), 7.70 (2H, d, 3′-H, 5′-H), 9.77 (1H, s, CHO);

^{13}C NMR (50.3 MHz, $CDCl_3$ see Table 3) δ 12.1 (q, C-4″), 20.14 (q, C-8), 26.33 (t, C-3″), 34.4 (t, C-2), 53.5 (d, C-2″), 54.3 (d, C-3), 68.9 (d, C-7), 83.9 (d, C-4), 112.7 (d, C-2′, C-6′), 124.8 (d, C-5), 126.0 (d, phenyl-C-4), 127.4 (s, C-4′), 128.1 (d, phenyl-C-2, C-6), 128.7 (d, phenyl-C-3, C-5), 132.3 (d, C-3′, C-5′), 134.1 (d, C-6), 139.3 (s, phenyl-C-1), 150.6 (s, C-1′), 173.0 (s, C-1″), 174.3 (s, C-1), 190.3 (d, CHO); EI-MS *m/z* 421 (M^+).

Dehydroobscurolide A_2 (**8**)

A solution of 55 mg obscurolide A_2 (**2**) and 360 mg MnO_2 in 5 ml acetone was stirred for 12 hours. After filtration and evaporation of the solvent *in vacuo*, the crude product was chromatographed on silica gel (column 40 × 2 cm, ethyl acetate - petroleum ether, 1 : 2). 45 mg (82%) **8** was obtained. MP 172°C; $[\alpha]_D^{20}$ +82.9°; Rf values 0.63 ($CHCl_3$ - MeOH, 9 : 1), 0.30 (ethyl acetate - petroleum ether, 1 : 1); IR (KBr) cm^{-1} 3500, 3360, 1765, 1680, 1610, 1540; UV λ_{max} (MeOH) nm (ε) 205 (18,200), 325 (25,500); ^1H NMR (200 MHz, acetone-d_6) see Table 2; ^{13}C NMR (50.3 MHz, acetone-d_6) see Table 3; EI-MS *m/z* 273 (M^+).

Acknowledgment

The authors wish to thank Mr. G. GREWE and Mrs. I. WERNER for technical assistance in fermentation and HPLC-diode array detection. The Fonds der Chemischen Industrie and the Deutsche Forschungsgemeinschaft (SFB 323) are gratefully acknowledged for financial support.

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