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DETECTION, ISOLATION AND STRUCTURE ELUCIDATION OF A CHLOROPHYLL *a* CATABOLITE FROM AUTUMNAL SENESCENT LEAVES OF *CERCIDIPHYLLUM JAPONICUM**†

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Key Word Index—*Cercidiphyllum japonicum*; Cercidiphyllaceae; chlorophyll *a* catabolism; chlorophyll *a* degradation; autumnal senescence; fall foliage; 19-formyl-1[21H,22H]bilinone.

Abstract—Extracts of autumnal leaves of the dicotyledonous, deciduous tree *Cercidiphyllum japonicum* cultivated at the Botanical Garden of Fribourg, Switzerland, were screened for chlorophyll catabolites by TLC utilizing the chromic acid degradation test. The constitution of the isolated material was elucidated by spectroscopy. The structure, an optically active bile-pigment-like 19-formyl-1[21H,22H]bilinone derivative, reveals that this compound originates from chlorophyll *a* and resembles the structures of previously isolated chlorophyll catabolites from the green alga *Chlorella protothecoides* and from the angiosperms, the monocot *Hordeum vulgare* and the dicot *Brassica napus*.

INTRODUCTION

During autumnal senescence, chlorophyll degradation occurs most impressively in the leaves of deciduous trees and shrubs. The disappearance of the chlorophylls is accompanied by the development of brilliant reds, yellows and golds. The apparent loss of this important chloroplast pigment marks an age-related deterioration of the photosynthetic capacity [3]. Progress has been achieved during the last five years in the elucidation of the molecular structures and the mechanisms involved in the degradation of the chlorophylls. The metabolites which proved to be linear tetrapyrroles were isolated from a chlorophyte *Chlorella protothecoides* Krüger [1], and from angiosperms of the families Gramineae (*Hordeum vulgare* L.) [4, 5], and Cruciferae (*Brassica napus* L.) [6, 7] (Fig. 1).

The green alga *C. protothecoides*, when grown heterotrophically under nitrogen starvation, excretes red chlorophyll *a* and *b* catabolites into the medium [1, 8, 9]. In contrast, excised primary leaves of barley (*H. vulgare*) aged artificially in darkness and cotyledons of the dicot rape (*B. napus*) cultivated under natural photoperiod, accumulate colourless tetrapyrrolic compounds in the vacuoles of the mesophyll cells; only catabolites of chlorophyll *a* have been found to date [10]. It was surprising to find that in all characterized catabolites regioselective oxidative ring scission occurred at the C(4)-C(5) *meso*-position with retention of C(5) as a formyl group on ring B, yielding 19-formyl-1[21H,22H] bilinone derivatives. *In vivo* labelling experiments performed with *C. protothecoides* cells unequivocally showed that a monooxygenase is involved in the primary cleaving step [11]. Most recently, we proved that the main red pigment **2c** isolated from the culture medium of the green alga can be transformed chemically in acidic medium, into a compound showing the same basic skeleton as the colourless breakdown products isolated from both barley and rape. Based on these studies, a generalized mechanism of enzymatic chlorophyll breakdown has been suggested [1, 2], linking the catabolite(s) isolated from the green alga to the catabolites found in higher plants.

We now report the detection, isolation and characterization of a new colourless chlorophyll *a* catabolite from autumnal leaves of the deciduous tree *Cercidiphyllum japonicum* which grows under natural conditions at the Botanical Garden of Fribourg, Switzerland.

RESULTS AND DISCUSSION

The constitution of the catabolite **1** isolated from *C. japonicum* was deduced from ^{1}H and ^{13}C NMR measurements and from mass spectra (see Experimental). FAB-mass spectrometry established molecular ions at *m/z* 645, 667 (base peak) and at 689, suggesting a

*Part 9 in the series 'Chlorophyll Catabolism'. For Part 8 see [1]. For a recent review see [2].

†Dedicated to Professor Horst Senger on the occassion of his 65th birthday.

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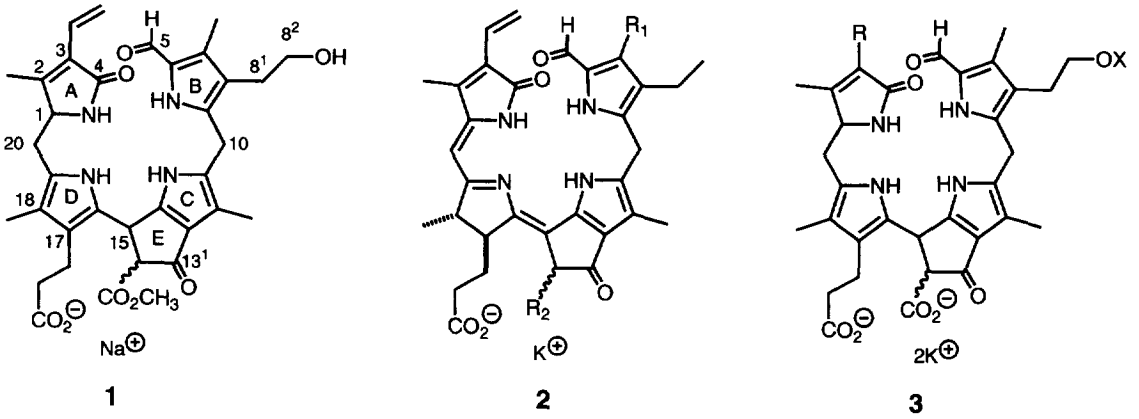

Fig. 1. Catabolites hitherto isolated from: (i) *Cercidiphyllum japonicum* Sieb. *et* Zucc. **1** [this work]; (ii) the unicellular green alga *Chlorella protothecoides* Krüger; **2a** $R_1 = CH_3$, $R_2 = COOK$; **2b**: $R_1 = CHO$, $R_2 = COOK$ (both characterized as dimethyl ester) [1, 8]; **2c**: $R_1 = CH_3$, $R_2 = H$ (characterized as free acid and as methyl ester) [9]; (iii) barley (*Hordeum vulgare* L.); **3a**: X = H, R = 1,2-dihydroxyethyl, as $O(13^4)$-methyl ester [4, 5]; (iv) rape (*Brassica napus* L.), **3b**: X = H, R = vinyl [7]; **3c**: X = malonyl, R = vinyl [6]; **3d**: X = 1-glucosidyl, R = vinyl [7].

tetrapyrrole moiety. Mixing of the sample with KI resulted in additional clusters, centred at *m/z* of 683 and 721, owing to ion exchange. Quantitative determination of sodium by flame emission spectrometry of the sample dissolved in MeOH showed the presence of equivalent amounts of sodium in the sample, which suggests the existence of a carboxylate group.

In the ^1H NMR spectrum of **1** dissolved in perdeuteromethanol, 33 of the 39 protons of the compound are seen as resonance signals, of which 19 protons account for one formyl, one vinyl, four methyls and one methoxycarbonyl group (Fig. 2). Connectivities in space are derived from $^1H\{^1H\}$ NOE difference experiments (Table 1; Fig. 2). Four amino, one hydroxyl and

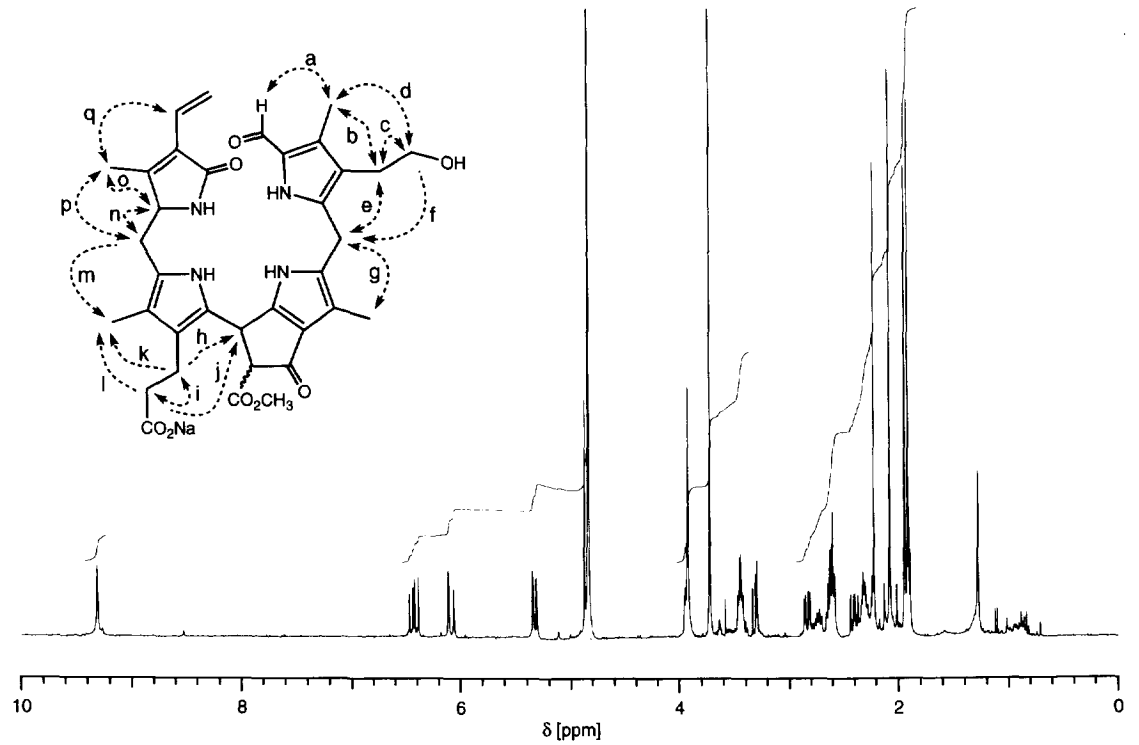

Fig. 2. 360 MHz ^1H NMR spectrum of **1** in CD_3OD showing the purity of the preparation. Owing to the acidity of the proton on $C(13^2)$ rapid H/D exchange occurs by the solvent. Therefore, the proton on C(15) at $\delta = 4.88$ ppm appears as a singlet (see text). In the structure the connectivities obtained from homonuclear NOE difference experiments are indicated by arrows (see Table 1).

Table 1. ^{1}H NMR signals of **1** assigned by ^{1}H{^{1}H} NOE difference experiments measured in CD_3OD (c 3×10^{-2} M)

Irradiated signals	Enhanced signal†	% Enhancement	Assignments in formula 1 (Fig. 2)
9.31	2.22 (3H, *s*, H-7^1)	5.0	a
6.43	5.33 (1H, *dd*, J = 11.6, J = 2.4, H_{cis}-3^2	8.7	Not shown
	1.94 (3H, *s*, H-2^1)	3.5	q
3.94*	2.84 (1H, *dd*, J = 14.2, J = 4.9, H_B-20)	1.2	n
	2.41 (1H, *dd*, J = 14.4, J = 9.5, H_A-20)	2.8	n
	1.94 (3H, *s*, H-2^1)	1.6	o
3.92*	2.68-2.55 (2H, m, H-8^1)	1.7	e
	2.08 (3H, *s*, H-12^1)	2.0	g
3.44	3.92 (2H, *s*, H-10)	Small	f
	2.68-2.55 (2H, *m*, H-8^1)	4.6	c
	2.22 (3H, *s*, H-7^1)	1.7	d
2.84*	3.97–3.89 (1H, m, H-1)	6.2	n
	2.41 (1H, *dd*, J = 14.4, J = 9.5, H_A-20)	14.8	Not shown
	1.94 (3H, *s*, H-2^1)	2.1	p
	1.91 (3H, *s*, H-18^1)	Small	m
2.74*	4.88 (1H, *s*, H-15)	7.8	h
	2.68-2.55 (1H, *m*, H_A-17^1)	4.2	Not shown
	2.36-2.26 (2H, *m*, H-17^2)	4.8	i
	1.91 (3H, *s*, H-18^1)	Small	k
2.61	3.92 (2H, *s*, H-10)	1.9	e
	3.48–3.42 (2H, *m*, H-8^2)	4.9	c
	2.80–2.68 (1H, *m*, H_B-17^1)	3.4	Not shown
	2.36–2.26 (2H, *m*, H-17^2)	1.6	i
	2.22 (3H, *s*, H-7^1)	1.9	b
	1.91 (3H, *s*, H-18^1)	Small	k
2.41*	3.97-3.89 (1H, *m*, H-1)	8.6	n
	2.84 (1H, *dd*, J = 14.2, J = 4.9, H_B-20)	13.9	Not shown
	1.91 (3H, *s*, H-18^1)	2.2	m
2.31*	4.88 (1H, *s*, H-15)	1.0	j
	2.80-2.68 (1H, *m*, H_B-17^1)	1.9	i
	2.68-2.55 (1H, *m*, H_A-17^1)	1.9	i
	1.91 (3H, *s*, H-18^1)	1.0	l
2.22	9.31 (1H, *s*, H-5)	2.6	a
	3.48–3.42 (2H, *m*, H-8^2)	Small	d
	2.68–2.55 (2H, *m*, H-8^1)	Small	b
2.08	3.92 (2H, *s*, H-10)	Small	g
1.94*	6.43 (1H, *dd*, J = 17.6, J = 11.7, H-3^1)	2.2	q
	3.97–3.89 (1H, *m*, H-1)	1.4	o
	2.84 (1H, *dd*, J = 14.2, J = 4.9, H_B-20)	Small	p

* The neighbouring signals are affected by off-resonance irradiation. The reported values are referred to 100% intensity of the sum of the saturated signals.

† The other resonance signals were assigned on the basis of their multiplicities and chemical shifts: δ 3.73 (3H, *s*, H-13^5); 6.09 (1H, *dd*, J = 17.6, J = 2.4, H_{trans}-3^2).

the acidic proton at C(13^2) rapidly exchange H/D in CD_3OD. Accordingly, no coupling between protons on C(15) and C(13^2) could be observed. In DMSO-d_6, as expected, the coupling (J = 3.4 Hz) has been established. Nevertheless, the latter spectrum was unsuitable for NOE measurements, because of serious signal overlappings. The longest wavelength in UV/visible spectrum at 312 nm excluded an extended conjugational system [12], but agreed with the presence of an α-formylpyrrole [13]. The absorption spectrum is almost identical with the spectrum published for **3a** by Kräutler *et al.* [5]. HR-mass spectrometry shows a molecular ion at m/z 667 $[M+H]^+$ and established the exact molecular mass to be 667.2740±0.0004, appropriate for $[C_{35}H_{40}N_4O_8Na]^+$ (calcd 667.2743) which is consistent with the NMR data for the constitution of sodium 3^1,3^2-didehydro-1,10,15,20,22,24-hexahydro-8^2-hydroxy-13^2-methoxycarbonyl-4,5-dioxo-4,5-seco-21H, 23H-phytoporphyrinate (**1**).

Neither the absolute nor the relative configurations of the three stereogenic centres at positions C(1), C(13^2) and C(15) are known; suitable crystals for X-ray structure analysis are not yet available. Nevertheless, the ease of H/D exchange on C(13^2) in ^{1}H NMR suggests that the small satellite peaks, for example at δ 2.00 and δ 9.27, originate from an equilibration of epimers in a ratio of 88:12. In ^{13}C NMR spectra, deuterium exchange at C(13^2) is also responsible for

the multiplet structure of the resonance found at δ 67.8 ppm. As already discussed for **3a** (see [4, 5]) the anti-configuration for the hydrogen atoms attached to C(13^2) and C(15) is assumed to be the predominant epimer, owing to steric hindrance between the A/D half of the tetrapyrrolic moiety and the ester group on C(13^2).

Signal splitting due to diasteriomeric mixtures at the stereogenic centres C(1), C(15) have not been observed by NMR. In contrast, the syn- and anti-diastereomers of analogue structures obtained from the main red chlorophyll degradation product **2c** of *C. protothecoides* by partial synthesis could be separated by preparative TLC. Both isolated diastereomers exhibit in ^{1}H NMR distinct differences in chemical shifts, and were found to be stable at the chiral centres [1]. The chiral centre C(1) is expected to racemize under alkaline conditions, as has been convincingly demonstrated in a series of structurally related 5(2H)-dipyrrylmethanones [14]. Consequently, strong alkaline conditions should be avoided during the isolation procedure. Compound **1** shows optical activity in solution and a well-structured circular dichroism spectrum, exhibiting several Cotton effects (Fig. 3). The curve is almost identical to the CD spectrum of the catabolite isolated from *H. vulgare* [5]. This agreement suggests that the chiral centres are equally configured, implying the presence of a set of similar enzymes in different orders of angiosperms.

The molecular structure **1** of the catabolite isolated from naturally aged *C. japonicum* leaves suggests the following catabolic processes: (i) oxygenolytic ring opening at the *meso*-position with simultaneous protanation of the methine-bridge C(10), (ii) saturation of the remaining methine bridge on C(1)-C(20), (iii) aromatization of the original pyrroline ring D by proton catalysed rearrangement and (iv) β-hydroxylation on C(8^2). The basic constitution is closely related to the structures of the catabolites isolated from *H. vulgare* and *B. napus* compounds **3a** and **3b–3d**, respectively (Fig. 1). An intact methoxycarbonyl group is present at the isocyclic ring E in the catabolite of *C. japonicum* and *H. vulgare*, suggesting that oxidative ring opening precedes methyl ester cleavage. Experiments with nonspecific estereases (hog liver esterease or lipase from *Candida cylindracea*), which had previously been applied for the methyl ester cleavage of the propionic side chain [15], failed to hydrolyse the C(13^2) ester group of compound **1**, implicating the requirement for a specific hydrolytic enzyme. All catabolites isolated to date from higher plants indicate the action of a specific enzyme(s), hydroxylating the nonactivated C(8^2) methyl group (Fig. 1), which is absent or inactive in the green alga *C. protothecoides*. This hydroxylation step increases the polarity of the catabolite, thereby facilitating the relocation of the catabolites into the vacuoles of the senescent plant cells [16]. No catabolites of chlorophyll *b* were observed in the extract from *C. japonicum* or found in *H. vulgare* and *B. napus*. Hence, it remains uncertain whether chlorophyll *b*, as suggested, is enzymatically converted in higher plants into chlorophyll *a* prior to ring opening [17, 18] or whether the chlorophyll *b* catabolite decomposes and/or eludes detection.

Degradation of the chlorophylls in plants serves presumably to avoid the accumulation of photodynamic active tetrapyrroles by interrupting the chromophoric system during the ageing process in which nutrients are relocated [19, 20]. However, 233 mg of a yellowish nonfluorescent compound were isolated from 250 g (fresh-weight) leaves of *C. japonicum*. The amounts

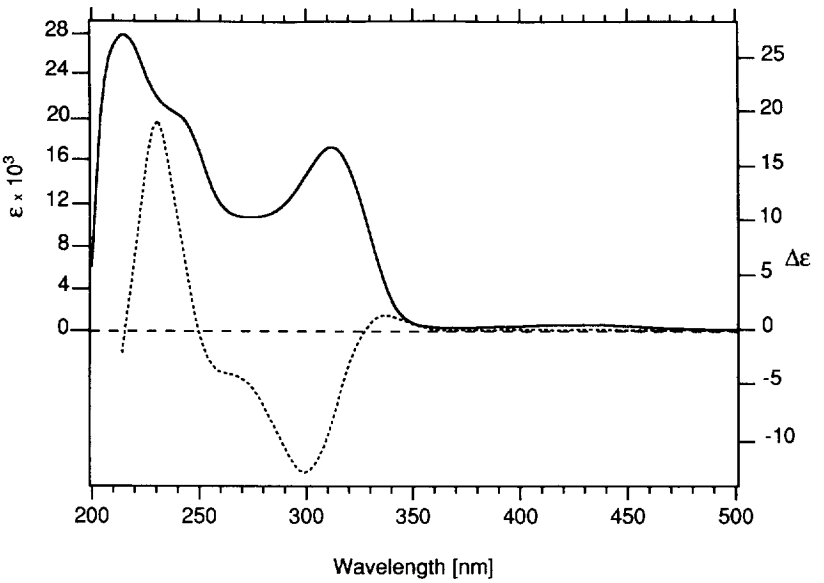

Fig. 3. Superimposed UV/visible (—) [$\varepsilon = f(\lambda)$] and CD (····) [$\Delta\varepsilon = f(\lambda)$] spectrum of the purified catabolite **1** in MeOH solution.

isolated agree quite well with the estimated content of 0.1–0.2% chlorophyll *a* generally found in fresh leaves of land plants [21], suggesting that chlorophyll catabolites are not further reutilized by the plant itself but rather (bio)degraded in the environment after leaf fall. Recently and independently from our work, Iturraspe *et al.* [22] reported the isolation of a structurally identical chlorophyll *a* catabolite from senescent leaves of *Liquidambar styraciflua* and *L. orientalis*. However, the chiroptical properties of this compound have not been reported.

Catabolism is generally associated with a series of chaotic destructive reactions under little control. Actually, the structures of the chlorophyll catabolites isolated from such diverse plant taxa as the Chlorophyceae, Gramineae, Cruciferae and Cercidiphyllaceae indicate a highly stereo-controlled process during catabolism, pointing to an orderly mechanism tightly controlled by enzymes.

EXPERIMENTAL

Materials. All chemicals were reagent grade; solvents were distilled prior to use. TLC aluminium foils precoated with silica gel 60 PF_{254} (0.2 mm) and preparative TLC plates coated with silica gel 60 $PF_{254+366}$ (1.25 mm thick, 20 × 20 cm) were purchased from Merck.

Spectra. ^1H NMR: 360.14 MHz with a Bruker AM-360 instrument supplied with an Aspect 3000 data system. Chemical shifts (δ) are given in ppm downfield from TMS, as int. standard, and coupling constants (J) in Hertz. ^{13}C NMR spectra were measured at 125.76 MHz with a Bruker Avance DRX-500 instrument using CD_3OD as int. standard (δ 49.0). MS and HR-MS were obtained with a Vacuum Generator Micromass 7070 E instrument equipped with a DS 11-250 data system using the FAB ionization in positive mode with Xe as primary atom beam at 7 keV and 1 μA. Samples were first dissolved in methanol (MeOH) and then added to the glycerol matrix. Accurate masses were obtained by internal matching with nearby matrix signals. Quantitative sodium-ion determination was performed by flame emission on a Video 12 spectrophotometer. NaI dissolved in MeOH was used for calibration, emissions were observed at 589 nm. UV/VIS spectra were recorded with a Hewlett Packard 8452A diode-array spectrophotometer, CD spectra were obtained with a Jobin-Yvon Auto Dichograph Mark V; $\Delta\varepsilon$[l mole^{-1} cm^{-1}]. Polarimetric measurements on a Perkin Elmer 241 MC instrument.

Plant material. Fresh yellow leaves were collected in September 1995 from the branches of a single male tree of *C. japonicum* Sieb. et Zucc., cultivated at the Botanical Garden Fribourg, Switzerland.

Detection of the catabolites. In order to detect colourless chlorophyll catabolites the 'on spot chromic acid degradation' technique was used, basically performed as suggested in [23], where bile-pigments are oxidized directly on TLC plates with chromic acid to the corresponding succinimides and/or maleimides. Accordingly, a spot of the CH_2Cl_2 extract obtained from the leaves of *C. japonicum* was overlaid with chromic acid soln. After developing the TLC plate in CH_2Cl_2–EtOAc–EtOH–HOAc (100:20:10:1) and staining, the presence of a pyrrolic compound was indicated by the appearance of blue-coloured spots. The position of the catabolite after developing of the spot on TLC in the first dimension using solvent systems of different polarities was found by application of the above procedure to the entire vertical lane. The corresponding spot, when irradiated at 254 nm, showed quenching of the fluorescent silica gel layer and was eventually isolated by prep. TLC. The purified compound assumed a rusty-like colour on the TLC plate when exposed to air and light after about two hr, as observed in [16] for the catabolite isolated from *H. vulgare*.

Isolation. 250 g (fresh wt) leaves of *C. japonicum* were mixed for 30 sec in a Waring Blender Model 32BL79 in a soln of Me_2CO–MeOH (1:1) (750 ml) at high speed without cooling. After centrifugation of the resulting slurry for 5 min at 5500 *g*, the pellets were washed with the same solvent mixt. (960 ml). After centrifugation, the combined supernatants were immediately filtered, concd to 250 ml by evapn of the solvents *in vacuo*, and then diluted with water (1000 ml). Extraction of the slightly turbid soln exhibiting pH 3 with CH_2Cl_2 (5 × 250 ml), drying of the extract with Na_2SO_4 and evapn of the solvent *in vacuo* yielded a residue which was filtered over a short chromatography column (9 cm high, 5 cm diameter, containing silica gel 40-63 μm) and eluted with CH_2Cl_2–MeOH (4:1). The collected frs were concd *in vacuo* and applied to 10 prep. TLC plates which were subsequently developed in $CHCl_3$–MeOH–H_2O (80:15:2). Extracting of the silica gel zone containing the catabolite with MeOH afforded after evapn of the solvent *in vacuo* a residue which was dissolved in MeOH–CH_2Cl_2 (1:1) (10 ml) and filtered over a degreased cotton plug. Evapn of the solvent *in vacuo* and drying of the solid in high vacuum at room temp. yielded 233 mg of a non-fluorescent, slightly yellow compound which was directly used for spectroscopic analysis. UV/VIS: λ_{max}^{MeOH} nm (log ε): 214 (4.44), 244 sh (4.29), 312 (4.23). CD: λ_{max}^{MeOH} nm ($\Delta\varepsilon$): 227 (+19.7), 252 sh (−4.0), 285 (−13.5), 316 (+1.6) (MeOH; c 4.80×10^{-2} mM). $[\alpha]_D^{20} = -152 \pm 1°$ (MeOH; *c* 0.0785). ^{13}C-DEPT-NMR (distortionless enhancement by polarization transfer (DEPT)) (CD_3OD): 8.9, 9.3 and 9.4 (C-2^1, C-7^1 and C-18^1), 12.5 (C-12^1), 22.5 (C-17^1), 23.9 (C-10), 28.1 (C-8^1), 30.6 (C-20), 37.3 (C-15), 40.5 (C-17^2), 52.9 (C-13^5), 62.2 (C-1), 62.7 (C-8^2), 67.8 (m, C-13^2) (multiplet due to H/D exchange with the solvent), 119.1 (C-3^2), 127.2 (C-3^1), 112.2, 115.6, 2 × 120.9, 124.4, 124.8, 125.8, 128.8, 129.4, 134.3, 135.5, 139.2, 156.9 and 161.8 (14 × C_{quat}), 171.9, 174.7 and 182.8 (lactam CO, ester COO and COO), 177.8 (CHO), 192.0 (CO). FAB-MS: *m/z* (%): 645 (17, $[M + 2H - Na]^+$), 667 (100, [M +

H]$^+$), 689 (56, [M + Na]$^+$). $R_f = 0.46$; $CHCl_3$–MeOH–H_2O (v/v/v, 80:15:2). ^1H NMR: Table 1.

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