Toxicity and Metabolism of the Chloral-Derived Mammalian Alkaloid 1-Trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo) in PC12 Cells[§]

Gerhard Bringmann[a,*], Doris Feineis[a], Miriam Münchbach[a], Ralf God[a], Karl Peters[b], Eva-Maria Peters[b], Rainald Mössner[c], and Klaus-Peter Lesch[c]

[a] Institut für Organische Chemie, Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany. Fax: (49) 931 888 4755. E-mail: bringman@chemie.uni-wuerzburg.de
[b] Max-Planck-Institut für Festkörperforschung, Heisenbergstraße 1, D-70506 Stuttgart, Germany
[c] Klinik und Poliklinik für Psychiatrie und Psychotherapie, Universität Würzburg, Füchsleinstraße 15, D-97080 Würzburg, Germany

* Author for correspondence and reprint requests

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Chloral-derived β-carbolines, which are structurally similar to the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, **5**), are discussed to contribute to neuronal cell death in idiopathic Parkinson's disease. The cytotoxicity of 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo, **4**) to neuronal-like clonal pheochromocytoma PC12 cells was examined by the determination of lactate dehydrogenase (LDH) release. After incubation for 48 h, **4** showed a strong dose-dependent cytotoxic activity towards PC12 cells with an ED_{50} value of 230 μM. In PC12 cells reductive dehalogenation of **4** was observed giving rise to the formation of 1-dichloromethyl-1,2,3,4-tetrahydro-β-carboline (**6**) as a main TaClo metabolite exhibiting a cytotoxic potential comparable to that of TaClo. An X-ray structure analysis, performed for the trifluoroacetyl derivative of **6**, revealed the *N*-substituent of such a highly chlorinated agent to be dramatically pushed out of the β-carboline ring 'plane' due to the high steric demand of the huge dichloromethyl group at C(1).

Key words: 1-Trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo), Rat Phaeochromocytoma (PC12) Cells, Cytotoxicity, Parkinson's Disease

Introduction

The β-carbolines comprise a group of tricyclic indole derivatives. Like other compounds, among them isoquinolines (McNaught *et al*., 1998; Naoi *et al*., 2002), pesticides (Betarbet *et al*., 2000), alkanes (Pezzoli *et al*., 2000) or heavy metals (Gorell *et al*., 1999), they have been considered as potential environmental inducers of neurodegenerative processes, due to their distinct neuropharmacological activities and their occurrence in mammalian organisms (Matsubara *et al*., 1998; Collins and Neafsey, 2000). 1-Trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo, **4**), which is structurally closely related to the well-established synthetic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, **5**) (Przedborski and Vila, 2001), is such a genuine neurotoxic agent for dopaminergic and serotonergic neurons (Bringmann *et al*., 1998; Riederer *et al*., 2002). TaClo (**4**) has seriously to be taken into account as a causative or supportive agent for the development of idiopathic Parkinson's disease, because it is able to severely affect the dopamine metabolism (Janetzky *et al*., 1999; Bringmann *et al*., 2000b; Riederer *et al*., 2002), to cause strong neuronal energy disturbances (Janetzky *et al*., 1999), and to induce DNA damaging (Bringmann *et al*., 2001) and apoptotic (Akundi *et al*., 2004) processes.

TaClo (**4**) is readily formed in the human organism by a Pictet-Spengler type condensation of the endogenously present biogenic amine tryptamine (Ta, **1**) and the non-natural aldehyde chloral (Clo, **3**) (Bringmann *et al*., 1999, 2002; Kochen *et al*., 2003). Its spontaneous formation can occur after application of the hypnotic chloral hydrate and upon exposition to the industrial solvent trichloroethylene (TRI, **2**), which is known to be metabolized to chloral (Fig. 1). TaClo (**4**) has been identified in concentrations ranging from less than 1 ng up to 70 ng per ml in blood samples obtained from patients who had been treated orally with chloral

[§] "Endogenous Alkaloids in Man", part 42; for part 41 see: Bringmann, Feineis, Brückner, God, Grote, and Wesemann (2006).

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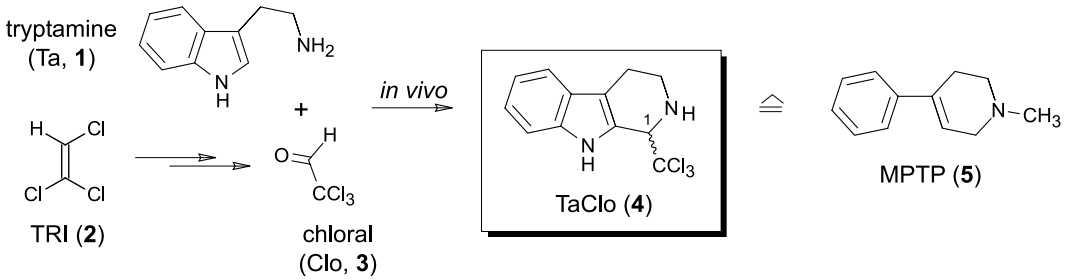

Fig. 1. Structural analogy of two neurotoxins acting on dopaminergic neurons: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, **5**), a well-established experimental tool in neurosciences, and the highly chlorinated 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo, **4**), a mammalian alkaloid formed from endogenously present tryptamine (Ta, **1**) and the hypnotic chloral (Clo, **3**) by Pictet-Spengler cyclization.

hydrate for three days up to five years (Bringmann *et al.*, 1999, 2002). The onset of Parkinson's disease in three chronically TRI-exposed persons showing a continuous release of TRI associated with the presence of TaClo on a ng-scale (Kochen *et al.*, 2003) gave further hints for the assumption that endogenously originating chloral-derived β-carbolines (such as **4**), even though formed only on a ng-scale, may chronically trigger progressive neurodegenerative lesions in brain that become manifest with age.

Clonal rat pheochromocytoma PC12 cells possess the ability to produce and secrete dopamine and can be induced to differentiate into neuronal-like cells. They are widely applied as a model system to study damaging and destructive processes in dopaminergic neurons leading to cell death. Cultures of PC12 cells have been used to investigate the mode of cell death caused by MPTP (**5**) and its bioactive metabolite 1-methyl-4-phenylpyridinium ion (MPP^+) (Hartley *et al.*, 1994).

The current report deals with the cytotoxicity of the chloral-derived tetrahydro-β-carboline TaClo (**4**) on PC12 cells as assessed by determining the activity of lactate dehydrogenase (LDH) in the culture medium relative to the total LDH activity (live plus dead cells). The effect of the duration of TaClo treatment (24, 48, and 72 h) on cytotoxicity was tested, too. Studies on TaClo biotransformation in PC12 cells revealed 1-dichloromethyl-1,2,3,4-tetrahydro-β-carboline (1-dichloromethyl-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole) (**6**) and 1,2,3,4-tetrahydro-β-carboline-1-carboxylic acid (1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole-1-carboxylic acid) (**7**) to be the main TaClo metabolites. The TaClo-derived compound **6** resulting from the reductive loss of one chlorine atom and the halogen-free heterocycle **7** were synthesized and examined for their cytotoxic properties towards PC12 cells using the LDH release assay. Furthermore, a comparative X-ray diffraction study was performed on the three-dimensional structures of the trifluoroacetyl derivatives of **4** and **6** to investigate the steric demand of their chlorine-containing substituents at C(1).

Results and Discussion

Exposure of PC12 cells to concentrations of TaClo (**4**) varying between 10 μM and 800 μM led to progessively enhanced degrees of cell death over 48-h incubations as indicated by lactate dehydrogenase (LDH) release. Cell viability was dose-dependently impaired with a moderate increase in LDH activity between 10 μM and 200 μM of **4**, while a nearly complete LDH release was observed after exposure to TaClo at concentrations beyond 600 μM (see Fig. 2). The increase in LDH release hints at a breakdown in membrane integrity, which could be due to a portion of necrotic cell death or could represent a later stage of apoptosis where cell membranes may become permeable. The assumption that TaClo can induce both, apoptosis and necrosis, in PC12 cell lines is in accordance with results from previous investigations (Akundi *et al.*, 2004) on TaClo cytotoxicity in the human neuroblastoma cell line SK–N–SH. In these dopaminergic cells, the lowest TaClo concentrations (150 μM) that cause significant cell death showed only a slight increase in LDH activity and predominantly induced apoptosis, whereas higher concentrations resulted in a significant LDH release and effected a shift of cell death towards necrosis (Akundi *et al.*, 2004).

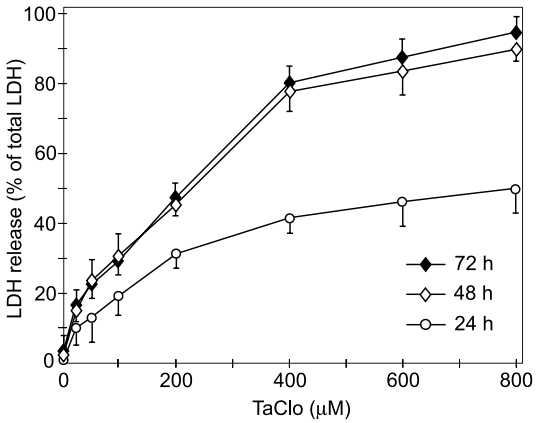

Fig. 2. Cytotoxicity of TaClo (**4**) to PC12 cells after treatment for 24 h (○), 48 h (◇), and 72 h (◆) assessed as percentage of lactate dehydrogenase (LDH) activity in the culture medium, relative to the total LDH activity (live plus dead cells). The data points represent means ± S.E.M. of triplicate measurements.

The effect of the duration of TaClo treatment (24, 48, and 72 h) on cytotoxicity towards PC12 cells was tested. As presented in Fig. 2, incubation with **4** for 24 h already showed a distinct cytotoxic effect, and after TaClo exposure for 48 h, a dose-related cytotoxicity (ED_{50} = 230 μM) was observed as described above. Treatment with **4** for 72 h did not significantly increase LDH release, indicating that full cytotoxicity is already obtained after 48 h. This concentration- and time-dependent mode of cell death had previously also been observed for other potent neurotoxins like MPP^+ and rotenone (Hartley *et al.*, 1994). The mechanism by which **4** induces toxic processes in PC12 cells is presumably associated with the production of reactive oxygen species (ROS), which cause damage and disruption of cellular membranes by lipid-peroxidation (Gerlach *et al.*, 1998). As reported earlier, TaClo (**4**) is able to trigger single-strand scissions to cell-free plasmid DNA when radical processes are initiated (Bringmann *et al.*, 2001). The radical formation is probably due to the chemically labile CCl_3 group, which may easily be attacked by ROS, with production of further radicals.

Metabolic processes affecting the trichloromethyl unit of TaClo (**4**) were studied more closely in PC12 cells applying liquid chromatography in combination with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). By means of neutral loss scanning, selected reaction monitoring (SRM) experiments were applied taking advantage of the elimination of a CH_2=NH fragment (−29 u) by a retro-Diels-Alder (RDA) reaction as typical of the tetrahydropyrido moiety of 1,2,3,4-tetrahydro-β-carbolines (Gutsche and Herderich, 1997). Most importantly, variable substituents at C(1) do not interfere with this diagnostic fragmentation. TaClo itself was clearly detected in PC12 cell medium by monitoring this characteristic loss of an imine fragment using the SRM experiments *m/z* 289 → *m/z* 260 (for [$^{35}Cl_3$]TaClo) and *m/z* 291 → *m/z* 262 (for [$^{35}Cl_2{}^{37}Cl$]TaClo) arising with nearly similar intensities of about 100:96 for a $^{35}Cl_3$ *vs.* a $^{35}Cl_2{}^{37}Cl$ portion (data not shown). Besides the identification of **4**, further molecular ions, *m/z* 255, *m/z* 257, and *m/z* 217 (see Fig. 3a) undergoing a neutral loss of 29 u, were recorded, hinting at the presence of additional tetrahydro-β-carbolines in PC12 cell medium. Product ion spectra of these putative TaClo metabolites (see Fig. 3b) as obtained by low-energy collision-induced dissociation (CID) showed characteristic fragmentation patterns indicative of 1-dichloromethyl-1,2,3,4-tetrahydro-β-carboline (**6**) and the halogen-free tetrahydro-β-carboline **7**. The RDA fragment *m/z* 188 (see Fig. 3b) being the most abundant product ion in the MS spectrum of the more rapidly eluting compound (see Fig. 3a) revealed the presence of the glyoxylate-derived heterocycle **7** resulting from **4** by complete – hydrolytic, non-reductive – dechlorination. Detection of the product ions *m/z* 226 and *m/z* 228 (see Fig. 3a) with the statistically expected intensity distribution of ca. 100:64 for [$^{35}Cl_2$]-**6** and [$^{35}Cl^{37}Cl$]-**6** corroborated the occurrence of 1-dichloromethyl-1,2,3,4-tetrahydro-β-carboline (**6**) arising from **4** by hydrodehalogenation. Based on this structural information as implied by tandem MS/MS, we synthesized authentic reference material. Identification of the two TaClo metabolites **6** and **7** was further unambiguously confirmed by comparison of their retention times, molecular ions, and product ion spectra with the respective data obtained from the synthetic compounds.

Interestingly, and in contrast to TaClo metabolism in rat organism (Bringmann *et al.*, 2000a), hydroxylation of the TaClo isocyle with subsequent formation of respective conjugates (such as glucuronidates) was not observed in PC12 cells at all. Furthermore, LC-MS/MS analysis did not give any hints for *N*-methylation products or for ring oxidation reactions leading to fully dehydrogenated compounds like norharman, nor for the occur-

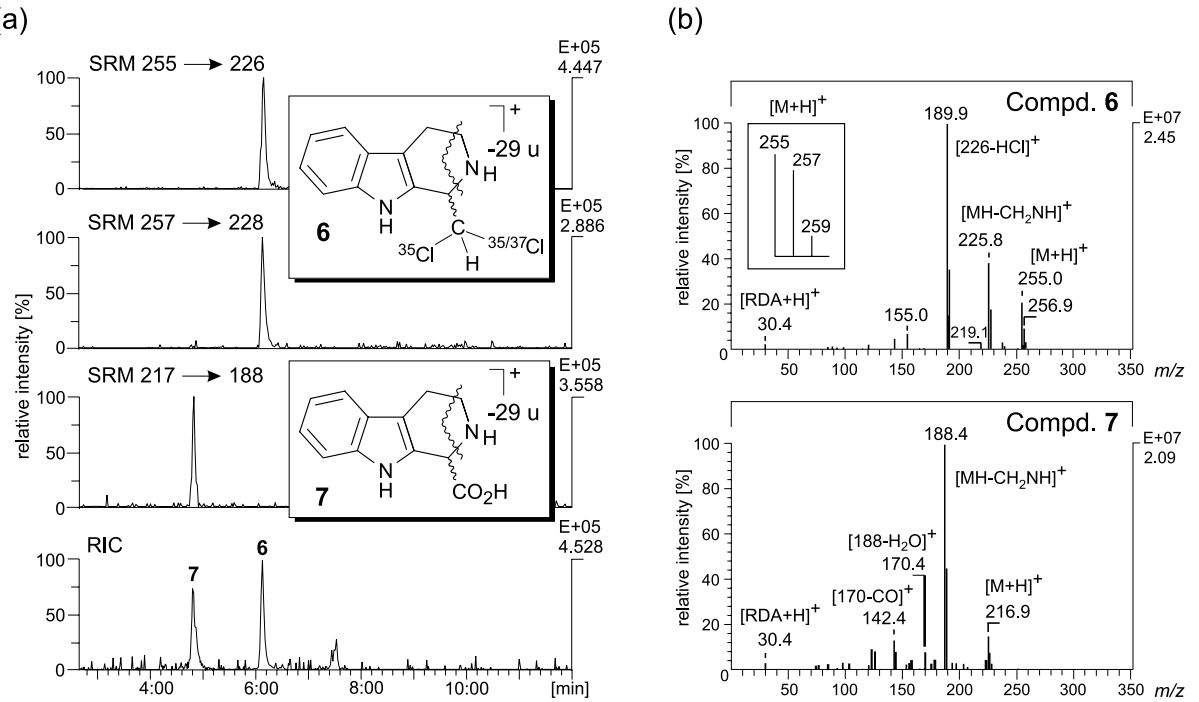

Fig. 3. Identification of the TaClo metabolites **6** and **7** in PC12 cell culture medium: (a) The recorded LC-MS/MS chromatogram is illustrated by the reconstructed ion current (RIC), and the ion traces obtained from SRM experiments (15 eV, 1.8 mTorr Ar) using the ion pairs *m/z* 255 → *m/z* 226 (for [^{35}Cl$_2$]-**6**), *m/z* 257 → *m/z* 228 (for [^{35}Cl^{37}Cl]-**6**), and *m/z* 217 → *m/z* 188 (for **7**). The inset structures show the origin of the product ions *m/z* 226/228 and *m/z* 188 originating from the protonated molecules of **6** and **7** by neutral loss of the CH_2=NH moiety (−29 u) by a RDA reaction. (b) Product ion spectra (15 eV, 1.8 mTorr Ar) of 1-dichloromethyl-1,2,3,4-tetrahydro-β-carboline (**6**) and 1,2,3,4-tetrahydro-β-carboline-1-carboxylic acid (**7**).

rence of 1-dichloromethylene-1,2,3,4-tetrahydro-β-carboline arising from TaClo by HCl elimination, which occurs smoothly *in vitro* (*e.g.*, in rat liver microsomes).

Cytotoxicity of **6** and **7** to PC12 cells was examined and compared to that of TaClo (**4**). In 48-h incubation tests, the chlorine-containing tetrahydro-β-carboline **6** was found to be cytotoxic to PC12 cells, whereas treatment with the halogen-free heterocycle **7** displayed only low toxicity (ca. 20% LDH release at 800 μM). Exposition to increasing concentrations of **6** (10–800 μM) showed a strong dose-dependent cytotoxicity (ED_{50} = 240 μM), which was nearly the same as that of **4** as indicated by LDH release (data not shown).

With respect to the strong cytotoxicity of chlorinated tetrahydro-β-carbolines towards dopaminergic cells and the strong interaction of these heterocycles with enzymes related to the energy metabolism and catecholamine biosynthesis (Bringmann *et al.*, 1998, 2000b; Janetzky *et al.*, 1999), the knowledge of the steric effects exhibited by voluminous chlorine-containing substituents at C(1) on the conformation of a tetrahydro-β-carboline ring system seemed desirable. For this reason, the three-dimensional structures of **4** and **6** were studied more closely by X-ray diffraction analysis.

In contrast to the authentic bioactive compound **6**, which unfortunately did not provide crystalline material, its trifluoroacetamide **8** did afford crystals well-suited for single-crystal structure determination (see Fig. 4a). This investigation revealed the tetrahydropyrido part to adopt a largely planarized half-chair conformation, with only C(3) and N(2) being located significantly out of the ring plane. The dichloromethyl substituent at C(1) is twisted out of the β-carboline ring plane, occupying a pseudo-axial position. The two chlorine atoms at C(14) show a perfectly staggered orientation with respect to the C(1)–C(14) bond, thus minimizing their steric interactions with C(13) and N(2). The influence of the large van der Waals ra-

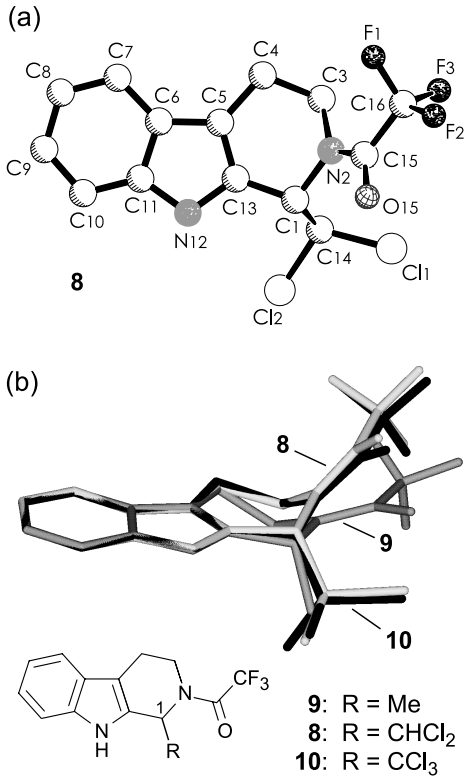

Fig. 4. (a) SCHAKAL plot of the crystal structure of *N*-trifluoroacetyl-1-dichloromethyl-1,2,3,4-tetrahydro-β-carboline (1-dichloromethyl-2-trifluoroacetyl-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole) (**8**) (viewed perpendicular) with a guide of the atomic numbering system adopted in the X-ray investigations. Arbitrarily, the enantiomer with the $CHCl_2$ group below the graphical plane is presented. (b) Joint plot of the structures of **8** (pale grey), the TaClo-derived *N*-trifluoroacetyl derivative **10** (black) (cf. Bringmann *et al.*, 2000b), and the eleagnine-related trifluoracetamide **9** (grey) (cf. Peters *et al.*, 1995) in the crystal, matched with respect to the indole part of the molecules, viewed horizontal to the tetrahydropyrido ring planes. All compounds are racemic in the crystal; for presentation, the enantiomers with the substituents at C(1) above the graphical plane have been chosen, arbitrarily. Hydrogen atoms have been omitted for reasons of clarity.

dius of the chlorine atom (1.8 Å) in comparison to the small size of the hydrogen atom (1.0 Å) on the conformation of the tetrahydropyrido moiety is best visualized by a matched plot (see Fig. 4b) of the crystal structures of **8** and the trifluoroacetamides **9** (from eleagnine) and **10** (from TaClo), which we have reported earlier (Peters *et al.*, 1995; Bringmann *et al.*, 2000b). Depending on the steric demand of the respective substitutent ($CH_3 < CHCl_2 \leq CCl_3$) at C(1), the trifluoroacetyl group at N(2) is increasingly pushed upwards: only slightly for the halogen-free 1-methyl-tetrahydro-β-carboline **9**, but dramatically for the 1-dichloro- and 1-trichloromethyl analogues **8** and **10**.

Summarizing, TaClo (**4**) and its metabolite 1-dichloromethyl-1,2,3,4-tetrahydro-β-carboline (**6**) showed a strong dose-dependent cytotoxicity against dopaminergic PC12 cells similar to the toxic effects exhibited by the neurotoxins MPP^+ and rotenone (Hartley *et al.*, 1994), whereas the cytotoxicity displayed by **7** was only moderate. As potent inhibitors of complex I of the mitochondrial respiratory chain, highly chlorinated tetrahydro-β-carbolines have been speculated to cause cell death presumably by necrosis due to the decline in ATP synthesis (Janetzky *et al.*, 1999). Previous studies on the cytotoxicity of **4** in the neuroblastoma cell line SK–N–SH, however, suggest that TaClo can induce both, apoptosis at low concentrations and necrosis at high concentrations (Akundi *et al.*, 2004). Current research is now focussing on a more detailed investigation concerning the mechanism by which TaClo and related compounds are able to initiate neuronal cell loss in PC12 cells. This work is in progress.

Materials and Methods

General

Melting points (uncorrected) were determined on a Reichert-Jung Thermovar hot-stage apparatus. Infrared spectra (IR) were obtained on a Perkin-Elmer Model 1420 spectrophotometer. KBr refers to a potassium bromide disk for infrared spectra. ^1H and ^{13}C NMR spectra were recorded on a Bruker AC 250 spectrometer at 250 MHz (for ^1H NMR) and 63 MHz (for ^{13}C NMR), respectively. Chemical shifts (δ) are reported in parts per million (ppm), and are referenced to internal methanol (^1H, δ = 3.33 ppm) or dimethylsulfoxide (^1H, δ = 2.50 ppm; ^{13}C, δ = 39.43 ppm) in the deuterated solvents. Coupling constants (J) are given in Hertz (Hz). The following abbreviations are used: s (singlet), d (doublet), m (multiplet), m_c (centered multiplet). Electron impact mass spectral data were obtained on a Finnigan MAT 8200 mass spectrometer. The peaks listed are those arising from the molecular ion $[M]^{+\cdot}$, those attributable to loss of certain fragments (M^+ minus a fragment), and some other prominent peaks. Elemental analyses were conducted by the Microanalysis

Laboratory of the University of Würzburg (Institute of Inorganic Chemistry) on a Carlo Erba Elemental Analyzer M 1106 apparatus.

Materials

All reagents used were of commercial quality. Organic solvents were dried and distilled prior to use. Foetal calf serum (FCS), horse serum, trypsin, and gentamicin were obtained from PAN Systems GmbH (Aidenbach, Germany). Dulbecco's PBS buffer and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL Life Technologies Ltd. (Paisley, UK). Tryptamine hydrochloride was purchased from Sigma (Deisenhofen, Germany), glyoxylate acid monohydrate and trifluoroacetic anhydride from Merck (Darmstadt, Germany). 1-Trichloromethyl-1,2,3,4-tetrahydro-β-carboline hydrochloride (TaClo · HCl, **4** · HCl) was synthesized from tryptamine and chloral in refluxing toluene as reported earlier (Bringmann and Hille, 1990). Reactions were monitored by thin-layer chromatography (TLC) on aluminum plates coated with silica gel 60 F_{254} (Merck). Column chromatography was performed on Merck silica gel (0.063–0.200 mm).

Syntheses

1-Dichloromethyl-1,2,3,4-tetrahydro-9*H*-pyrido-[3,4-*b*]indole hydrochloride (**6** · HCl)

A solution of **4** · HCl (500 mg, 1.70 mmol) in methanol (120 ml) was degassed *in vacuo*, and saturated with hydrogen. After addition of 40 mg of 10% palladium on charcoal as hydrogenation catalyst, the reaction mixture was stirred at room temperature for 90 min, and then filtered through Celite to remove the catalyst. The residue obtained after evaporation of the solvent was purified by column chromatography on silica gel (eluent: *tert*-butyl methyl ether) yielding a dark-green solid. Crystallization from methanol saturated with hydrochloric acid gave the title compound (230 mg, 0.79 mmol, 47% yield) as an amorphous green-colored crystalline powder, m.p. 220 °C (dec). – IR (KBr): υ = 3220 (m, indole NH), 2990 (CH), 1540, 1450, 1430, 1400, 800 (s, CCl), 740 cm^{-1}. – ^{1}H NMR (CD_3OD): δ = 3.02–3.18 (m, 2H, 4-H), 3.53–3.64 (m, 1H, 3-H), 3.84–3.92 (m, 1H, 3-H), 5.55 (m_c, 1H, 1-H), 6.93 (d, J = 3.1 Hz, 1H, $CHCl_2$), 7.07–7.13 (m, 1H, 6-H or 7-H), 7.18–7.25 (m, 1H, 6-H or 7-H), 7.38–7.42 (m, 1H, 5-H or 8-H), 7.51–7.55 (m, 1H, 5-H or 8-H). – ^{13}C NMR [$(CD_3)_2SO$]: δ = 17.8 (C-4), 42.1 (C-3), 58.8 (C-1), 70.4 ($CHCl_2$), 108.7, 111.6, 118.4, 119.2, 122.5, 124.8, 125.2, 136.3. – EI-MS (rel. int.) [ion assignment]: (m/z) = 258/256/254 (0.5/4.0/5.6) [M^+], 220/218 (1.5/3.5) [M^+ – HCl], 184 (11) [M^+ – 2 Cl], 183 (10) [M^+ – HCl – Cl], 171 (100) [M^+ – $CHCl_2$]. – $C_{12}H_{12}Cl_2N_2 \cdot HCl$: calcd. C 49.43, H 4.49, N 9.61; found C 49.88, H 4.61, N 9.62.

1-Dichloromethyl-2-trifluoroacetyl-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole (**8**)

To a suspension of **6** · HCl (500 mg, 1.72 mmol) in dry dichloromethane (50 ml), trifluoroacetic anhydride (0.6 ml, 900 mg, 4.29 mmol) was added dropwise at 0 °C. The mixture was stirred at room temperature for 2 h, and concentrated *in vacuo*. Purification of the residue by crystallization from methanol provided the title compound (475 mg, 1.36 mmol, 79% yield) as pale-green crystals, suitable for X-ray structure analysis, m.p. 194 °C (dec). – IR (KBr): υ = 3320 (s, indole NH), 3050, 2980, 2900, 2840 (CH), 1680 (s, C=O), 1445, 1190, 1170, 1145 (s, CN, CF), 790 (s, CCl), 745 cm^{-1}. – ^{1}H NMR (CD_3OD): δ = 2.93–3.02 (m, 2H, 4-H), 3.82–3.94 (m, 1H, 3-H), 4.26–4.33 (m, 1H, 3-H), 6.01 (d, 1H, 1-H or $CHCl_2$), 6.13 (d, 1H, 1-H or $CHCl_2$), 7.14–7.20 (m, 1H, 6-H or 7-H), 7.25–7.31 (m, 1H, 6-H or 7-H), 7.40–7.44 (m, 1H, 5-H or 8-H), 7.52–7.55 (m, 1H, 5-H or 8-H). – ^{13}C NMR [$(CD_3)_2SO$]: δ = 23.0 (C-4), 42.6 (C-3), 45.1 (C-1), 53.5 ($CHCl_2$), 109.5 (CF_3), 112.3, 119.1, 120.4, 123.2, 127.5, 129.7, 138.2. – EI-MS (rel. int.) [ion assignment]: (m/z) = 354/352/350 (0.9/4.8/7.5) [M^+], 316/314 (2.0/6.5) [M^+ – HCl], 279 (27) [M^+ – HCl – Cl], 267 (100) [M^+ – $CHCl_2$], 169 (26) [267 – $COCF_3$ – H], 154 (45). – $C_{14}H_{11}Cl_2F_3N_2O$: calcd. C 47.89, H 3.16, N 7.98; found C 47.95, H 3.06, N 7.82.

1,2,3,4-Tetrahydro-9*H*-pyrido[3,4-*b*]indole-1-carboxylic acid (**7**)

The title compound was synthesized according to a published procedure (Peura and Nousiainen, 1981), yet with a slight modification: tryptamine hydrochloride (147 mg, 0.75 mmol) and glyoxylic acid monohydrate (76 mg, 0.82 mmol) were dissolved in water (10 ml) by warming on a steam bath to approximately 45 °C. Subsequent addition of 150 μl of 10% aqueous sodium hydroxide resulted in the precipitation of the title compound. Stirring of the reaction mixture was continued for 2 h. The pure crystalline material was filtered off

and dried over silica gel in a vacuum desiccator providing **7** (137 mg, 0.63 mmol, 85% yield) as colorless crystals, m.p. 212 °C [lit. (Peura and Nousiainen, 1981) 205–208 °C]. – IR (KBr): υ = 3380 (m, OH), 3275 (s, indole NH), 3025, 2860, 2760 (CH), 2370, 1620, 1590, 1450, 1390, 1220, 740 cm^{-1}. – ^1H NMR [$(CD_3)_2SO$]: δ = 2.71–2.98 (m, 2H, 4-H), 3.48–3.67 (m, 2H, 3-H), 4.74 (s, 1H, 1-H), 6.95–7.08 (m, 2H, 6-H and 7-H), 7.36 (d, $^4J_{5,6}$ = 8.3 Hz, 1H, 5-H), 7.55 (d, $^3J_{8,7}$ = 8.3 Hz, 1H, 8-H), 10.7 (s, 1H, CO_2H). – ^{13}C NMR [$(CD_3)_2SO$]: δ = 18.0 (C-4), 40.6 (C-3), 55.20 (C-1), 99.5, 103.9, 110.6, 112.4, 126.1, 129.1, 131.0, 165.5 (CO_2H). – EI-MS (rel. int.) [ion assignment]: *m/z* = 216 (2.9) $[M]^+$, 172 (60) [M – CO_2], 171 (100) [M – CHO_2], 169 (72) [M – CH_3O_2], 155 (37) [M – CH_3NO_2]. – $C_{12}H_{12}N_2O_2$: calcd. C 66.65, H 5.59, N 12.96; found C 66.46, H 5.47, N 12.67.

Crystal structure determination

The single crystal of **8** chosen for X-ray investigations was a pale-green prism. All measurements of diffraction intensities were performed at room temperature on a BRUKER AXS P4 four-circle diffractometer with an incident beam graphite monochromator (Mo–Kα radiation, λ = 0.71073 Å) in ω-scan mode and $2\theta_{max}$ = 55° (Siemens, 1996). The unit cell parameters were determined by least-squares refinement using 15 centered reflections. The structure was solved by direct-phase determination and refined by full-matrix anisotropic least-squares with the aid of the program package SHELXL-97 (Sheldrick, 1997). In refinements, weights were used according to the scheme $w = 1/[\sigma^2(F_o)]$. The hydrogen positions were calculated using a riding model and were considered fixed with isotropic thermal parameters in all refinements. In the crystal of **8**, the molecules are connected by intermolecular hydrogen bonds H(12)···O(15) (2.07 Å) to form a zigzag chain parallel to [100]. Software used to prepare material for publication was SCHAKAL 88 (Keller, 1990).

The details of the crystal structure determination and refinement for **8** are given in Table I. The crystallographic data (excluding structure factors) have been deposited with the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK [fax: (+ Int) 44-1223 336 033; e-mail: deposit@ccdc.cam.ac.uk]. A complete listing of the atomic coordinates can be obtained free of charge, on request, on quoting the depository no. CCDC-291493, the names of the authors, and the journal citation.

Table I. Crystal data and structure refinement for **8**.

Empirical formula	$C_{14}H_{11}Cl_2F_3N_2O$
Molecular mass [g mol^{-1}]	351.16
Crystal system	monoclinic
Space group	$P2_1/n$
a [Å]	16.208(5)
b [Å]	11.079(3)
c [Å]	8.627(2)
β [°]	100.88(2)
Volume [$Å^3$]	1521.3(7)
Formula units per cell	$Z = 4$
D_{calcd} [g cm^{-3}]	1.533
Crystal size [mm]	$0.40 \times 0.55 \times 0.35$
Scan mode	Wyckoff-scan
θ-range [°]	$1.75 \le \theta \le 27.5$
Range in *hkl*	$0 \le h \le 21$
	$0 \le k \le 14$
	$-11 \le l \le 11$
Reflections collected	3900
Unique reflections	3493
Reflections with $F > 3\sigma(F)$	2749
Lin. absorption coeff. [mm^{-1}]	0.46
Absorption correction	ψ-scan
Data/restraints/parameters	3493/0/204
Data-to-parameter ratio	13.48
Goodness-of-fit on F^2	1.042
Final agreement factors	R = 0.055, Rw = 0.054
Weighting scheme for Rw	$w = 1/\sigma^2 (F_o)$
Largest difference peak [$eÅ^{-3}$]	0.43
Largest difference hole [$eÅ^{-3}$]	0.50

Cell culture

Rat pheochromocytoma (PC12) cells from American Type Culture Collection (ATCC; CRL-1721) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% foetal calf serum, and 30 mg l^{-1} gentamicin at 37 °C under a humified 95% air and 5% CO_2 atmosphere. Stock cultures of PC12 cells were maintained in 500-ml culture flasks. Medium was changed every 3–4 d, and the cells were subcultured every 7–10 d. Confluent cultures were routinely harvested by trypsinization (0.05%) and split at 1:10. For all experimental purposes, cells were seeded on petri dishes (50 mm in diameter) at an initial density of 10^6 cells in 4 ml of the above medium. About 24 h after plating on petri dishes, confluent PC12 cells were incubated in fresh growth medium and exposed to TaClo (10, 50, 100, 200, 400, 600, and 800 μM) at 37 °C for 24, 48, and 72 h. In a second series of tests, confluent PC12 cells were treated in the same manner with the

TaClo metabolites **6** and **7** for 48 h. Therefore, solutions of TaClo (100 mM) or its metabolites **6** (100 mM) and **7** (100 mM) in dimethylsulfoxide (DMSO) were sterilized, then diluted in growth medium to reach the desired concentrations, and finally added to the cultures, after filtration through 0.2-μm syringe filters (Whatman Inc., Clifton, NJ, USA).

Measurement of LDH release

Lactate dehydrogenase (LDH) release was quantified using the Sigma cytotoxicity detection kit according to the manufacturer's instructions. At the given times, medium was removed from individual culture wells. Cell monolayers were dislodged by addition of 100 μl of 1% Triton X-100 in PBS (Sigma) and centrifuged for 5 min at $500 \times g$ at room temperature. Cell death was assessed by determining the activity of released lactate dehydrogenase (LDH, EC 1.1.1.27) relative to the total LDH activity. To measure released LDH, the culture medium was aspirated, while total LDH was determined in the culture medium after addition of Triton X-100 to suspend adhered cells. The supernatants were mixed with LD-L reagent (Sigma) and the increase in absorbance of NADH at 340 nm was measured. Linearity of the assay was established with Lintrol, a commercially available LDH standard (Sigma). All LDH assays were performed in triplicate and mean values (± S.E.M.) were calculated.

Preparation of PC12 culture medium samples for LC-MS/MS analysis

Workup by solid-phase extraction (SPE) was accomplished on C-18 sorbent Bakerbond SPE-cartridges (200 mg of sorbent) using a Baker SPE 12-G system vacuum chamber (J. T. Baker, Philipsburg, PA, USA). Cartridge pretreatment was carried out by flushing with methanol (2 ml) and Milli-Q water (2 ml). The conditioned C-18 cartridge was loaded with PC12 culture medium (2 ml) and, after gentle vacuum aspiration, washed with water (3 ml). The analytes were eluted by passing methanol (3 ml) through the column. After syringe filtration using a 0.22-μm filter (Waters), the solvent was evaporated to dryness in a Speed Vac vacuum concentrator (Savant, Vanves, France). The purified samples were redissolved in aqueous 0.5% trifluoroacetic acid (0.5 ml), and a 5-μl volume was directly injected onto the LC-MS/MS system. Each HPLC run of a PC12 culture medium sample was followed by a water–methanol injection to achieve an optimal regeneration of the HPLC column.

High performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-MS/MS)

All chromatographic separations were performed on a Symmetry C-18 column (dimensions: 150×2.0 mm; particle size: 5 μm) from Waters (Eschborn, Germany). As mobile phases, acetonitrile (A) and demineralized (B) water were used. B was acidified to pH 3 with 0.1% trifluoroacetic acid. The HPLC system used was an Applied Biosystems dual-syringe pump model 140B (Bai, Bensheim, Germany) equipped with a Rheodyne valve fitted with a 5-μl loop. The separation of PC12 culture medium samples was carried out with a flow of 300 μl/min. A nonlinear solvent gradient was programmed starting with 10% A and 90% B (1 min) to 100% A within 12 min. ESI-MS was performed on a Finnigan-triple stage quadrupole (Q1q2Q3) TSQ 7000 instrument (Finnigan MAT, Bremen, Germany) equipped with a pneumatically assisted electrospray interface. Data acquisition and postprocessing were done on a Personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) with ICIS 8.1 software. Nitrogen was used both as the sheath (70 p.s.i.; 1 p.s.i. = 6894.76 Pa) and the auxiliary gas (10 units), argon as the collision gas. The electrospray capillary voltage was 4 kV, and the temperature of the heated inlet capillary, serving simultaneously as a repeller electrode (20 V), was maintained at 250 °C. Positive ions were detected by scanning from 150 to 500 u with a total scan duration of 1.0 s for a single spectrum. Product ions were monitored by selecting the protonated precursors $[M + H]^+$ in Q1, performing collision-induced dissociation (CID) in the collision cell q2, and detecting the product ions in Q3. MS/MS experiments were performed at a collision gas pressure of 1.8 mTorr (1 Torr = 133.322 Pa), and collision energies ranging from 15 to 35 eV (laboratory frame of reference) scanning a mass range from 20 to 350 u with a total scan duration of 3.0 s. The mass spectra were measured in the centroid mode and referenced to 100% intensity of the base peak. From the resulting characteristic MS/MS fragmentation patterns of **4**, **6**, and **7**, the following most

abundant ion pairs were chosen for selected reaction monitoring (SRM) experiments: *m/z* 289 → *m/z* 260 and *m/z* 291 → *m/z* 262 (for **4**), *m/z* 255 → *m/z* 226 and *m/z* 257 → *m/z* 228 (for **6**), and *m/z* 217 → *m/z* 188 (for **7**). These ion pairs represent the protonated molecules $[M + H]^+$ and the product ions resulting from the cleavage of a CH_2=NH fragment (–29 u) (cf. Fig. 3).

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