Tetrahydro-β-carbolines, Potential Neuroactive Alkaloids, in Chocolate and Cocoa

Tomas Herraiz*

Instituto de Fermentaciones Industriales, Spanish National Research Council, Juan de la Cierva 3, 28006, Madrid, Spain

INTRODUCTION

Tetrahydro-β-carbolines (THβCs) (1,2,3,4-tetrahydro-9H-pyrido(3,4-b)indole) are naturally occurring indole alkaloids produced from indoleamines and aldehydes and/or alpha-ketoacids through Pictet–Spengler condensation (Figure 1). Tetrahydro-β-carbolines and β-carbolines have attracted the attention of neurochemists who have pointed out their occurrence under physiological conditions in biological tissues and fluids (Buckholz, 1980; Airaksinen and Karl, 1981; Melchior and Collins, 1982; Rommelspacher et al., 1991; Adachi et al., 1991; Brossi, 1993; Callaway et al., 1994). This has encouraged speculation on their putative role in the central nervous system where they could function as neuromodulators. THβCs inhibit the monoamine oxidase and the monoamine uptake, and bind to the benzodiazepine receptor (Buckholz, 1980; Airaksinen and Karl, 1981; Braestrup et al., 1980; Melchior and Collins, 1982; Myers, 1989; Rommelspacher et al., 1991; Cox and Cook, 1995). Simultaneously, tetrahydro-β-carbolines have been increasingly studied in relation with alcoholism where they might play a role in the etiology or addiction (Rozin et al., 1991) and active substances that might be involved in any physiological behavior responsible for cravings (Di Tomaso et al., 1996b; Gustche and Herderich, 1997).

Chocolate is a popular food, enjoyed largely for its great sensory properties. One of the most pleasant effects of eating chocolate is the “good feeling” that many people experience. However, chocolate craving is still incompletely understood (Rozin et al., 1991) and active research has attempted to find possible unknown bioactive substances that might be involved in any physiological behavior responsible for cravings (Di Tomaso et al., 1996). Chocolate contains several biologically active constituents (methylxanthines, biogenic amines, and cannabinoïd-like substances), all of which potentially cause abnormal behavior and psychological sensation that parallel those of other addictive substances (Bruinsma and Taren, 1999). In this regard, this paper reports the identification and occurrence of novel THβCs neuroactive alkaloids in chocolates and cocoas, and briefly discusses their origin and possible biological implications.

* Fax: 34-91-5644853. E-mail: ifiht16@ifi.csic.es.
Tetrahydro-β-carbolines in Chocolate Products

MATERIALS AND METHODS

**Reference Compounds and Samples.** L-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) was purchased from Sigma Chemical Co. (St. Louis, MO) and synthesized from L-tryptophan and acetaldehyde, and two diastereoisomers (1S,3S, major compound and 1R,3S, minor compound) (Brossi et al., 1973; Herráiz and Ough, 1993). 1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid (THCA) and 1-ethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (ETCA) were synthesized from L-tryptophan, and formaldehyde and propionaldehyde, respectively (Herráiz, 1997). 6-Hydroxy-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (6-OHMTH/C) was synthesized from serotonin oxide (Sigma) and acetaldehyde, whereas 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTH/C) was synthesized from tryptamine (Sigma) and acetaldehyde by a Picott–Spengler condensation. Data of NMR, MS, and GC–MS (trimfluoroacetyl and methoxycarbonyl methyl ester derivatives) were consistent with the structures of the synthesized compounds (Herráiz and Ough, 1994; Herráiz, 1997, Herráiz and Sanchez, 1997).

Commercial samples of dark chocolate (50–85% cocoa), milk chocolate (up to 30% cocoa), commercial cocoa powders, and chocolates breakfast cereals were purchased in local supermarkets and subsequently analyzed for TH/Cs as indicated below.

**Isolation of Tetrahydro-β-carbolines.** TH/Cs in chocolate products and cocoa were isolated using a SCX–solid-phase extraction method (Adam and et al., 1991; Herráiz et al., 1993). Aliquots of 2–5 g were homogenized in 0.6 M HClO4 (15–20 mL) containing 1 mg/mL of semicarbazide and centrifuged (5000g, 10–15 min, 0–5 °C). An aliquot of supernatant was derivatized with 6 mL of ETCA solution (5 mg/L) used as internal standard (IS), and loaded onto benzensulfonic acid-derivatized silica SCX columns (Bond Elut, 3 mL size, Varian, Harbor City, CA). After they were washed with 6 mL of 0.1 N HCl, 2 mL of methanol, and 6 mL of HPLC water, and rinsed with 2 mL of 0.4 M phosphate buffer (pH 9.1), the TH/Cs were eluted with 4 mL of methanol with 0.4 M phosphate buffer pH 9.1 (1:1). The eluates were injected into the HPLC.

**Chromatographic and Quantitative Analysis.** Chromatographic analysis was performed using an HPLC with a 1046A fluorescence detector and a 3365-Series II HP Chemstation (Hewlett-Packard, Santa Clara, CA). A 150 mm × 3.9 mm, 5 μ, Novapak C18 column (Waters) was used for HPLC separation. Fluorescence detection was carried out at 270 nm for excitation and 343 nm for emission. Eluents were 50 mM ammonium phosphate buffer adjusted to pH 3 with phosphoric acid (Eluent A) and 20% of A in acetonitrile (Eluent B). Two gradients were used; first, 0% B to 32% B in 8 min, then 90% B at 18 min, and 100% B at 20 min; and second, 0% B to 75% B in 60 min. The flow rate was 1 mL/min, the oven temperature was 40 °C, and the injection volume was 20 μL.

Quantitative analysis of TH/Cs in chocolate and cocoa was calculated from calibration curves obtained with standard solutions of known concentration against ETCA used as an internal standard, and carried through the entire isolation procedure. For quantitation of MTCA, the same response factor of MTCA (Sigma) was used for its two diastereoisomers. All this evidenced the presence of TH/Cs in chocolate products. A further unequivocal proof was obtained by HPLC–MS. Extracted samples of chocolate and cocoa gave patterns of fluorescence spectra in good agreement with those afforded by standards. This is illustrated in Figure 3 for 6-OHMTH/C, 1-methyl-1,2,3,4-tetrahydro-β-carboline (THCA), 1-ethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) in both diastereoisomers, and 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTH/C) (Figure 2b). TH/Cs isolated from chocolate and cocoa gave patterns of fluorescence spectra in good agreement with those afforded by standards. This is illustrated in Figure 4 for OMT/3-COOH and MTH/C.

RESULTS

TH/C alkaloids, along with their respective amino acid and amine precursors (i.e., L-tryptophan, serotonin, and tryptamine), were successfully separated by RP–HPLC (Figure 2a). The possible existence of these alkaloids in chocolate and cocoa was studied in SCX-extracts which provided chromatographic peaks coeluting with 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (6-OHMTH/C), 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCA), 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) in both diastereoisomers, and 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTH/C) (Figure 2b). TH/Cs isolated from chocolate and cocoa gave patterns of fluorescence spectra in good agreement with those afforded by standards. This is illustrated in Figure 3 for OMT/3-COOH and MTH/C.

The identity of TH/Cs was confirmed by HPLC retention time and co-injection with authentic standards. Also, the excitation and emission spectra of the HPLC peaks were compared with those of TH/Cs standards to check peak purity. To achieve that, eluting peaks were trapped into the fluorescence-detector flow cell by stopping the solvent pump, and excitation and emission spectra were recorded.

**Chemical Identification by HPLC–MS.** Samples of chocolate and cocoa (25 g) were homogenized with 0.6 M HClO4 containing semicarbazide (60 mg/mL), centrifuged (12000 rpm, 15 min, 0 °C), and SCX-extracted as described above. The eluting fractions corresponding to phosphate buffer and methanol (1:1) were evaporated under vacuum (less than 40 °C) and redissolved in the same buffer prior to HPLC–MS. Chemical identification was accomplished by HPLC–MS on a 3.9 mm × 150 mm Novapak C18 column (Waters), by using an HPLC–MSD series 1100 (Hewlett-Packard) (electrospray, positive-ion mode). Conditions: eluents A, formic acid (0.5%); B, 0.5% formic acid in acetonitrile; 60% B in 60 min; flow, 0.7 mL/min; cone voltage, 50 V; mass range, 50–600 amu.

**Oxidation of TH/C-3-COOH and Chromatographic Analysis.** The samples isolated from SCX were injected into the HPLC and the peaks corresponding to TH/C-3-COOH (THCA and MTCA) were collected. They were treated with a Na2Cr2O7 solution (80 °C for 1 h), then basified, and extracted with CH2Cl2. Under these conditions TH/Cs-3-COOHs are oxidized to their corresponding β-carbolines (Herráiz, 2000). The organic solvent was evaporated under a He stream, redissolved in 0.1M HCl, and injected into RP–HPLC under the same chromatographic conditions as above except for detection (excitation 245 nm, emission 445 nm). β-carbolines were trapped into the detection cell and the spectral characterization accomplished as for TH/C-3-COOHs.
from 0.01 to 0.85 μg/g and not detected to 0.21 μg/g, respectively. The content of TH/Cs (6OHMTH/C, MTCA, and MTH/C) was generally higher in dark chocolate than in commercial cocoa powders, milk chocolate, and cereals containing chocolate. This seemed to be correlated with the total content of cocoa in the samples. However, the content of THCA was generally higher in cereals containing chocolate than in the rest of the samples. Table 1 also lists the content of the two biogenic amines serotonin and tryptamine. Serotonin, which is the presumable precursor of 6OHMTH/C after condensation with acetaldehyde, averaged 2.9, 0.58, 1.25, and 0.095 μg/g for chocolates, milk chocolates, cocoa, and cereals containing chocolate. Tryptamine, a presumable precursor of MTH/C averaged 0.83, 0.16, 0.69, and 0.04 μg/g for the same products, respectively.

**DISCUSSION**

To the best of my knowledge this is the first report on TH/C alkaloids in chocolate. Four compounds were identified and subsequently quantified: 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (6OHMTH/C), 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCA), 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (13S and 1R,3S diasteroisomers) (MTCA), and 1-methyl-1,2,3,4-tetrahydro-β-carboline (MTH/C). Chocolates, cocoa, and chocolate-containing cereals contained varying amounts of each compound ranging from undetectable to several μg/g. Within a report of an improved chromatographic method of carbolines, Tsuchiya et al. (1996a) analyzed MTH/C in a sample of cacao and reported a higher concentration than those found here. This discrepancy is probably due to differences in the samples. We have previously reported that several foods and fermented alcoholic beverages contain appreciable amounts of two of those TH/Cs found in chocolates, THCA and MTCA, reaching up to several mg/kg (Herraiz et al., 1993, Herraiz, 1996–2000). Interestingly, the concentration of THCA and MTCA in chocolate and cocoa is comparable to that of alcoholic beverages such as wine, beer, and liquor, which contain a relatively high amount of those compounds. The origin of these tetrahydro-β-carbolines is a reaction involving L-tryptophan and aldehydes that are present or otherwise released during the processing of foods and beverages. Its chemical formation depends on the amount of precursors, storage time, pH, temperature, and processing conditions (Herraiz and Ough, 1993; Herraiz, 1996). The same reaction is likely to occur in chocolates that suffer a fermentation from cacao beans and heating processes. Then, it is expected that serotonin, L-tryptophan, and tryptamine afford the corresponding TH/Cs (6OHMTH/C, MTCA, and MTH/C) through a Pictet–Spengler condensation with acetaldehyde (see Figure 1).

The biological significance of tetrahydro-β-carbolines and β-carbolines is related to their potential pharmacological actions on the nervous system, playing a role as neuromodulators via effects on monoamine oxidase (MAO), biogenic amine (serotonin) uptake/release, and benzodiazepine receptor binding. Then, these compounds exogenously supplied, or hypothetically produced in vivo, might become bioactive, exhibiting behavioral and/or toxicological implications. In this regard, it is very likely that part of the β-carbolines found in the human tissues and fluids have a dietary origin. Although the concentration of TH/Cs in foods is usually
On the other hand, the existence of a relationship between alcohol consumption and tetrahydro-β-carboline alkaloids in chocolate (among them phenylethylamine, tryptamine, and serotonin) has deserved much attention in the past (Mutos, 1987). Lankford and Myers (1994) reported that the relative low concentration (i.e. an average of 30 g/person/day of total THCs) in chocolate, and also their presence in many other foods including fruit products (Herraiz et al., 1993, Herraiz, 1996). Also, because THCs are a novel group of potential pharmacologically active substances in chocolate. Despite their supposed relative low concentration (i.e. an average of 30 g/person/day consumption of dark chocolate would account for an ingestion of up to 0.21 mg/person/day of total THCs), the presence of THCs exhibiting potential bioactive or neuroactive properties could play a role in craving, and this hypothesis deserves further attention. However, against this assumption are both the relatively low levels of these alkaloids in chocolate, and also their presence in many other foods including fruit products (Herraiz, 1996, 1998). Also, because THCs related ß-carbolines are mild inhibitors of MAO, then, they could hypothetically potentiate the effect of amines in chocolate (among them phenylethylamine, tryptamine, serotonin, and others) (Baker et al., 1987).

Table 1. Concentration (µg/g) of Serotonin, Tryptamine, and Tetrahydro-β-carboline Alkaloids in Chocolates, Cocoas, and Chocolate-Containing Cereal Products

<table>
<thead>
<tr>
<th>Substance</th>
<th>Dark Chocolate (n = 10)</th>
<th>Milk Chocolate (n = 4)</th>
<th>Cocoa Powder (n = 8)</th>
<th>Chocolate Cereals (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
<td>SD</td>
<td>Range</td>
<td>x</td>
</tr>
<tr>
<td>Serotonin</td>
<td>2.90</td>
<td>1.55</td>
<td>1.37–5.08</td>
<td>0.586</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>0.83</td>
<td>0.33</td>
<td>0.2–1.16</td>
<td>0.16</td>
</tr>
<tr>
<td>THCA</td>
<td>0.34</td>
<td>0.12</td>
<td>0.23–0.68</td>
<td>0.10</td>
</tr>
<tr>
<td>SS-MTCA</td>
<td>1.74</td>
<td>0.24</td>
<td>1.37–2.0</td>
<td>0.512</td>
</tr>
<tr>
<td>RS-MTCA</td>
<td>0.69</td>
<td>0.11</td>
<td>0.53–0.88</td>
<td>0.195</td>
</tr>
<tr>
<td>MTHC</td>
<td>0.13</td>
<td>0.064</td>
<td>0.05–0.21</td>
<td>0.056</td>
</tr>
</tbody>
</table>

Figure 4. Reconstructed ion chromatograms of an extract of chocolate analyzed by HPLC–MS (electrospray). Chromatographic conditions: Novapak C18 column; eluent A, 0.5% HCOOH; eluent B, 0.5% HCOOH in acetonitrile; 60 min 60% B; 40 °C.

not excessively high, the successive ingestion of these compounds during the diet would surely increase the THC/Cs in the body.

Chocolate is usually described as a craved food, and although the hedonic appeal of chocolate (fat, sugar, texture, and aroma) is likely to be the predominant factor, it also has been pointed out that it may possibly contain potential pharmacologically active substances responsible for the craving (Bruinsma and Taren, 1999). In this regard, several compounds have been considered, such as phenylethylamine, methylylantin, and the recently reported anandamide (Di Tomaso et al., 1996). THC/Cs are a novel group of potential pharmacologically active substances in chocolate. Despite their supposed relative low concentration (i.e. an average of 30 g/person/day consumption of dark chocolate would account for an ingestion of up to 0.21 mg/person/day of total THC/Cs), the presence of THC/Cs exhibiting potential bioactive or neuroactive properties could play a role in craving, and this hypothesis deserves further attention. However, against this assumption are both the relatively low levels of these alkaloids in chocolate, and also their presence in many other foods including fruit products (Herraiz, 1996, 1998). Also, because THC/Cs related ß-carbolines are mild inhibitors of MAO, then, they could hypothetically potentiate the effect of amines in chocolate (among them phenylethylamine, tryptamine, serotonin, and others) (Baker et al., 1987).

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