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Theacrine (1,3,7,9-tetramethyluric acid) synthesis in leaves of a Chinese tea, kucha (*Camellia assamica* var. *kucha*)

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Abstract

Theacrine (1,3,7,9-tetramethyluric acid) and caffeine were the major purine alkaloids in the leaves of an unusual Chinese tea known as kucha (*Camellia assamica* var. *kucha*). Endogenous levels of theacrine and caffeine in expanding buds and young leaves were ca. 2.8 and 0.6–2.7% of the dry wt, respectively, but the concentrations were lower in the mature leaves. Radioactivity from *S*-adenosyl-L-[methyl-^{14}C]methionine was incorporated into theacrine as well as theobromine and caffeine by leaf disks of kucha, indicating that *S*-adenosyl-L-methionine acts as the methyl donor not only for caffeine biosynthesis but also for theacrine production. [8-^{14}C]Caffeine was converted to theacrine by kucha leaves with highest incorporation occurring in expanding buds. When [8-^{14}C]adenosine, the most effective purine precursor for caffeine biosynthesis in tea (*Camellia sinensis*), was incubated with young kucha leaves for 24 h, up to 1% of total radioactivity was recovered in theacrine. However, pulse-chase experiments with [8-^{14}C]adenosine demonstrated much more extensive incorporation of label into caffeine than theacrine, possibly because of dilution of [^{14}C]caffeine produced by the large endogenous caffeine pool. These results indicate that in kucha leaves theacrine is synthesized from caffeine in what is probably a three-step pathway with 1,3,7-methyluric acid acting an intermediate. This is a first demonstration that theacrine is synthesized from adenosine via caffeine.

Keywords: *Camellia assamica*; Theaceae; Kucha; Chinese tea; Biosynthesis; Caffeine; Purine alkaloids; 1,3,7,9-Tetramethyluric acid; Theacrine

1. Introduction

As a part of research to discover new Chinese tea resources for beverage and medicinal uses, Ye et al. (1997,1999) found some tea plants grown in China accumulated large quantities of different purine alkaloids than normal tea (*Camellia sinensis* L.) leaves which contain mainly caffeine (Fig. 1). Leaves of cocoa tea (*C. ptilophylla*), for instance, contains primarily theobromine, which is present in *C. sinensis* in low amounts as it is converted to caffeine. The biosynthesis and metabolism of purine alkaloids in leaves of cocoa tea have been investigated by Ashihara et al. (1998). Kucha (*C. assamica* var. *kucha*) is another unusual tea because, as well as caffeine, its leaves contain sizeable amounts of theacrine (1,3,7,9-tetramethyluric acid) (Ye et al., 1999).

Since theacrine was discovered as a minor component of *C. sinensis* leaves by Johnson (1937), it has been detected along with related methyluric acids, such as liberine (*O*(2), 1,9-trimethyluric acid) and methylliberine (*O*(2),1,7,9-tetramethyluric acid) (Fig. 1) in several species of coffee including *Coffea liberica, C. dewevrei* and *C. abeokuta* (Wanner et al., 1975; Baumann et al., 1976; Petermann and Baumann, 1983), as well as seeds of various *Herrania* and *Theobroma* species (Baumann and Wanner, 1980; Hammerstone et al., 1994). Petermann and Baumann (1983) demonstrated that caffeine was converted to liberine via theacrine and methylliberine in leaves of the three *Coffea* species referred to above.

In the present study, the levels of purine alkaloids in kucha leaves at different developmental stages were determined. Theacrine was found in leaves at all stages of development although its concentration decreased

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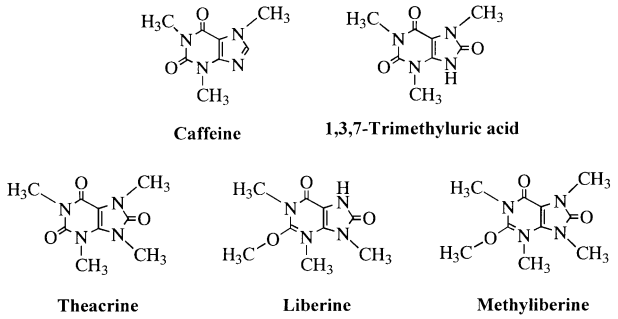

Fig. 1. Structures of caffeine and methyluric acids.

with age. Other methyluric acids, such as liberine and methylliberine, were not detected. The biosynthesis of theacrine was investigated using ^{14}C-labelled adenosine, caffeine and *S*-adenosyl-L-methionine (SAM). The data obtained indicate that caffeine is synthesized in kucha leaves by the same pathway that is utilised in *C. sinensis* and *C. arabica,* and it is then converted, by hydration, oxidation and methylation, to theacrine.

2. Results and discussion

2.1. Endogenous purine alkaloids in kucha

Table 1 indicates that the major purine alkaloids accumulating in kucha leaves are theacrine and caffeine together with a small amount of theobromine. No theophylline, liberine or methylliberine was detected. These results confirm preliminary data obtained with young leaves and shoots of kucha (Ye et al., 1999). The findings presented in Table 1 show high purine alkaloid levels in expanding buds made up of ca. 40% caffeine 40% theacrine, and 20% theobromine. In young and mature leaves theacrine represented ca. 60% of total purine alkaloids. We also examined the profile of purine alkaloids in old leaves from near the base of trees. Although the content of individual leaves was variable, smaller amounts (0.1–0.5% of dry wt) of theacrine and little or no caffeine were usually detected.

Petermann and Baumann (1983) demonstrated that theacrine was further converted to liberine possibly via methylliberine in aged leaves of *C. dewevrei* var. *excelsa*, *C. liberica* and *C. abeokutae*. However, methylliberine and liberine were not detected in any of the kucha leaf samples that were analysed in the present study.

2.2. Methyl donor for theacrine synthesis

It is well established that SAM is the methyl donor for the three methylation steps in the caffeine biosynthetic pathway in *C. sinensis* (see Suzuki et al., 1992; Suzuki and Waller, 1988; Waller et al., 1999; Ashihara and Crozier, 1999, 2001). The data presented in Table 2 show that [methyl-^{14}C]SAM is readily incorporated into caffeine, theobromine, the immediate precursor of caffeine, and theacrine in kucha leaves (Table 2). Although the incorporation of label from [methyl-^{14}C]SAM into theacrine was the highest in young leaves, it still occurred in mature leaves. This raises the possibility that caffeine is further methylated and converted to theacrine. The methylation of the N-*9* position of theacrine is probably performed with SAM as the methyl donor as shown at the N-*1*, N-*3* and N-*7* positions of caffeine in conversions catalysed by specific *N*-methyltransferases (see Kato et al., 2000; Ashihara and Crozier, 2001). This possibility could not be investigated further because of the limited availability of fresh kucha leaves and technical difficulties in preparing active cell-free systems which precluded detailed in vitro enzymatic studies.

2.3. Biosynthesis of theacrine from adenosine

It has recently been established that adenosine is a major precursor of the caffeine biosynthetic pathway in leaves of *C. sinensis*. As mentioned in the previous section, the formation of caffeine from xanthosine is closely associated with the SAM-cycle (also known as the activated methyl-cycle), because the three methylations in the caffeine biosynthetic pathway use SAM as a methyl donor. During this process, SAM is converted to *S*-adenosyl-L-homocysteine which in turn is hydrolysed

Table 1
Levels of endogenous purine alkaloids in expanding buds, young and mature leaves of kucha (*Camellia assamica* var. *kucha*) plants

Common name	Chemical name	Expanding buds	Young leaves	Mature leaves
Theobromine	3,7-Dimethylxanthine	14.5±1.0 (21)	3.5±1.0 (9.6)	1.2±0.4 (5.8)
Caffeine	1,3,7-Trimethylxanthine	27.0±1.5 (39)	5.5±2.0 (15)	7.0±1.6 (34)
Theophylline	1,3-Dimethylxanthine	nd (–)	nd (–)	nd (–)
Theacrine	1,3,7,9-Tetramethyluric acid	28.0±3.5 (40)	27.5±9.0 (75)	12.5±1.7 (60)
Liberine	*O*(2),1,9-Trimethyluric acid	nd (–)	nd (–)	nd (–)
Methylliberine	*O*(2),1,7,9-Tetramethyluric acid	nd (–)	nd (–)	nd (–)
Weight	Dry weight (mg/leaf)	7.32±1.5	45.9±4.9	284±5.2

Data are expressed as mg per g dry wt and presented as mean values±standard deviation ($n=3$). The values in parentheses show the percentage of total purine alkaloids.
nd, Not detected.

Table 2
Incorporation of radioactivity from 9 μM [methyl-^{14}C]*S*-adenosyl-L-methionine (specific activity 2.15 GBq/mmol) into purine alkaloids in young and mature leaves of kucha plants

Purine alkaloids	Young leaves	Mature leaves
Theobromine	0.03±0.01 (5.2)	0.08±0.02 (6.0)
Caffeine	0.06±0.01 (10.3)	0.02±0.01 (1.5)
Theacrine	0.04±0.01 (6.9)	0.07±0.01 (5.2)
Total uptake	0.58±0.06 (100)	1.34±0.05 (100)

Duration of incubation was 18 h. The mean total uptake of radioactivity and incorporation of radioactivity into various purine alkaloids by the segments of expanding buds or young leaves are presented as kBq/100 mg fr. wt±standard deviation ($n=3$). The figures in parentheses are values expressed as a percentage of total radioactivity taken up.

to L-homocysteine and adenosine. The adenosine is converted to xanthosine and used to synthesize the purine ring of caffeine while L-homocysteine is recycled to replenish SAM which is the source of the methyl groups required for caffeine biosynthesis (Ashihara and Crozier, 2001; Koshiishi et al., 2001). Incorporation of radioactivity from [8-^{14}C]adenosine into purine alkaloids in expanding buds and young leaves of kucha was, therefore, investigated. After a 24 h-incubation, radioactivity was incorporated into theobromine, caffeine and theacrine (Table 3). In buds, 0.5% of the radioactivity taken up was associated with theacrine while an 0.9% incorporation was observed in young leaves. This suggests the rate of purine alkaloid biosynthesis in kucha is high in young tissues as has been demonstrated with *C. sinensis* (Fujimori et al., 1991; Ashihara et al., 1997), *C. arabica* (Fujimori and Ashihara, 1994; Ashihara et al., 1996) and *Ilex paraguariensis* (Ashihara, 1993).

To investigate the sequence of theacrine biosynthesis from adenosine, a pulse-chase experiment was carried out with [8-^{14}C]adenosine. The data obtained are presented in Table 4. After an 8 h-pulse, most radioactivity was recovered as nucleotides (41%) and nucleic acids (43%), and very little label was associated with either theobromine 1.09%), caffeine (0.08%) or theacrine (0.00%). During a 3 day chase experiment, the radioactivity in nucleotides and nucleic acids declined, $^{14}CO_2$ increased, radioactivity associated with theobromine changed little (1.09–1.33%) while incorporation into caffeine increased to over 4%. After a 16 h chase radioactivity from [8-^{14}C]adenosine was detected in theacrine (0.1%) but this did not increase to any extent with further incubation. The low incorporation of radioactivity into theacrine may be due to dilution of the specific activity of [^{14}C]caffeine being synthesized by the very large endogenous caffeine pool.

These patterns of incorporation of label into nucleotides, $^{14}CO_2$, theobromine and caffeine with kucha leaves are similar to those obtained with *C. sinensis* leaves (Ashihara and Crozier, 1999). Caffeine biosynthesis from adenosine in the two species, therefore probably occurs by the same "adenosine→AMP→IMP→XMP→xanthosine→7-methyl-xanthosine→7-methylxanthine→theobromine → caffeine" pathway.

2.4. Conversion of caffeine to theacrine

The pattern of metabolism of [8-^{14}C]adenosine was in keeping with the conversion of caffeine to theacrine despite the relatively low incorporation of radioactivity into methyluric acid. To further investigate this point, metabolism of [8-^{14}C]caffeine to theacrine and other potential methyluric acids was investigated in segments of kucha buds and leaves. During the course of a 4 h incubations 7.8% of the [8-^{14}C]caffeine taken up by kucha bud segments was converted to theacrine, with a lower incorporation in young leaves (Table 5).

2.5. Overall metabolism of [8-^{14}C]caffeine

Because caffeine appeared to be an intermediate of theacrine synthesis in kucha, metabolism of [8-^{14}C]caffeine was examined using mature leaf segments and the data obtained are presented in Table 6. Most of the exogenously supplied [8-^{14}C]caffeine remained unmetabolised. Small amounts of radioactivity were recovered as theacrine, theobromine, ureides (allantoin plus allantoic acid), CO_2 and an unknown metabolite which arguably could be an intermediate between caffeine and theacrine.

Table 3
Incorporation of radioactivity from 10 μM [8-^{14}C]adenosine (specific activity 1.85 GBq/mmol) into purine alkaloids in expanding buds and young leaves of kucha plants

Purine alkaloids	Expanding buds	Young leaves
Theobromine	0.18±0.09 (3.0)	1.2±0.03 (7.4)
Caffeine	0.17±0.05 (2.8)	1.0±0.09 (6.2)
Theacrine	0.03±0.003 (0.5)	0.14±0.02 (0.9)
Total uptake	6.1±0.3 (100)	16.2±4.2 (100)

Duration of incubation was 24 h. The mean total uptake of radioactivity and incorporation of radioactivity into various purine alkaloids by the segments of expanding buds or young leaves are presented as kBq/100 mg fr. wt±standard deviation ($n=3$). The figures in parentheses are values expressed as a percentage of total radioactivity taken up.

Table 4
Overall metabolism of 10 μM [8-^{14}C]adenosine (specific activity 1.85 GBq/mmol) in pulse-chase experiments with mature leaves of kucha plants

Metabolites	8 h (pulse)	16 h (chase)	40 h (chase)	64 h (chase)
Nucleotides	41.04±4.01	30.36±2.31	23.02±0.53	21.81±1.28
Theobromine	1.09±0.06	1.33±0.25	1.12±0.05	1.07±0.15
Caffeine	0.08±0.08	2.14±0.08	2.71±0.09	4.08±0.01
Theacrine	0.00±0.00	0.10±0.04	0.13±0.01	0.11±0.03
Ureides	0.03±0.03	0.29±0.04	0.20±0.00	0.22±0.01
Unknown metabolites	6.34±0.71	11.86±0.49	11.10±0.40	11.96±1.04
Nucleic acids	43.30±3.77	23.11±0.73	22.47±1.43	18.39±0.51
CO_2	5.86±0.51	30.83±3.98	39.24±2.21	42.38±2.04
Total uptake	10.30±0.32	10.81±0.75	9.97±1.14	9.96±0.62

Leaf segments (ca. 100 mg fr. wt) were incubated with [8-^{14}C]adenosine for 8 h (pulse), and then the incubation medium was replaced by fresh medium without tracer. The radioactivity was chased for a further 16, 40 and 64 h. The incorporation of radioactivity is expressed as percentage of total radioactivity taken up by the leaves. Total uptake is expressed as kBq/100 mg fr. wt. Average values±standard deviation ($n=3$) are shown.

Table 5
Conversion of 9 μM [8-^{14}C]caffeine (specific activity 1.96 GBq/mmol) to theacrine in expanding buds and young leaves of kucha plants

Metabolites	Expanding buds	Young leaves
Theacrine	0.16±0.06 (7.6)	0.07±0.02 (2.6)
Total uptake	2.1±0.14 (100)	2.7±0.14 (100)

Duration of incubation was 4 h. The incorporation of radioactivity into theacrine and total uptake of radioactivity in the segments of expanding buds or young leaves expressed as kBq/100 mg fr. wt±standard deviation ($n=3$). The figures in parentheses represent data expressed as a percentage of total radioactivity taken up by expanding buds or young leaves.

Table 6
Overall metabolism of 9 μM [8-^{14}C]caffeine (specific activity 1.96 GBq/mmol) in pulse-chase experiments with mature leaves of kucha plants

Metabolites	8 h (pulse)	16 h (chase)
Residue caffeine	95.80±0.01	92.81±0.60
Theacrine	0.46±0.06	0.90±0.06
Theobromine	0.52±0.01	0.88±0.04
Ureides	0.10±0.01	0.00±0.00
Unknown metabolite	0.28±0.06	0.12±0.12
CO_2	0.11±0.01	2.51±0.33
Total uptake	5.86±0.07	3.70±0.11

Leaf segments (ca. 100 mg fr. wt) were incubated with [8-^{14}C]caffeine for 8 h (pulse), and then the incubation medium was replaced by fresh medium without tracer. The radioactivity was chased for a further 16 h. The incorporation of radioactivity is expressed as percentage of total radioactivity taken up by the leaves. Total uptake is expressed as kBq/100 mg fr. wt. Average values and standard deviation ($n=3$) are shown.

The data, indicate that at least part of the theacrine pool in kucha originates from caffeine but the participation of other precursors cannot be eliminated at this juncture. The most likely intermediate between caffeine and theacrine is 1,3,7-trimethyluric acid (Fig. 1). Although other possibilities do exist, most of the compounds involved are not established plant products.

In addition to theacrine, there was conversion of [8-^{14}C]caffeine to ureides and $^{14}CO_2$, which in *C. sinensis* occurs by demethylation to xanthine and metabolism via the conventional purine catabolic pathway (Ito et al., 1997; Ashihara and Crozier, 1999). Theobromine also formed from [8-^{14}C]caffeine in mature kucha leaves, in amounts similar to theacrine (Table 6). In *C. sinensis,* small amounts of methylated xanthines derived from theophylline, undergo salvage and are converted to theobromine which is a precursor, rather than a degradation product of caffeine (Ashihara et al., 1997).

2.6. Conclusion

In kucha, theacrine was an major purine alkaloid in buds and young leaves. Young leaves incorporated [8-^{14}C]adenosine into theacrine via caffeine while a low level of conversion of [8-^{14}C]caffeine to theacrine was shown to occur in expanded buds and young and mature leaves. Traditionally, kucha leaves have been used as a cold cure, and there is also a belief that

drinking kucha tea enhances longevity. However, further study is required to determine whether there is any substance to these claimed medicinal roles and whether or not theacrine and other purine alkaloids are involved in these putative effects. The isolation of the kucha genes encoding enzymes for theacrine synthesis and their insertion into *C. sinensis,* would open up the possibility of producing genetically-modified tea that is rich in theacrine yet contains low levels of caffeine. Such a beverage would appeal to the general public who are consuming decaffeinated tea and coffee in increasing amounts (Ashihara and Crozier, 2001).

3. Experimental

3.1. Plant materials

Expanded buds and leaves used in this study were obtained from field-grown trees of kucha (*C. assamica*, var. *kucha* Chang et Wang) at the experimental farm of the Zhongshan University, Guangzhou, China. Samples were collected in October 2000 and in May 2001. The developmental stages of the leaves were categorized as (i) expanding buds, (ii) young leaves and (iii) mature leaves. Young leaves were the most recently emerged small size first and second leaves from new shoots. Mature leaves comprised the fully expanded fourth or fifth leaf below the apex from newly emerged shoots.

3.2. Chemicals

[8-^{14}C]Adenosine and [8-^{14}C]caffeine were purchased from Moravek Biochemicals Inc. (Irvine, CA, USA) and *S*-adenosyl-[methyl-^{14}C] L-methionine (SAM) was obtained from Amersham International plc. (Amersham, Bucks., UK). Authentic samples of theacrine, liberine and methylliberine were kindly supplied by Thomas W. Baumann, University of Zurich, Switzerland. Other methylxanthines and methyluric acids were purchased from Sigma (St. Louis, MO, USA).

3.3. Determination of purine alkaloid content

Purine alkaloids were extracted from leaves (100 mg) with ca. 10 ml methanol using a mortar and pestle. The homogenate was centrifuged at 12,000 × *g* for 15 min, and supernatant was evaporated. After complete evaporation of the methanol, extracts were dissolved in distilled water (5 ml) and the purine alkaloid content of 5–10 μl aliquots was determined by HPLC analysis. This was carried out with a Shimadzu LC 10A HPLC system (Kyoto, Japan), using a ferruleless column (250 mm×4.6 mm i.d.), packed with a 5 μm ODS Hypersil support (Shandon, Runcorn, Cheshire, UK), eluted a flow rate of 1 ml/min with a 25 min, 0–40% gradient of methanol in 50 mM sodium acetate, pH 5.0. This reversed phase HPLC system separates methylxanthines and methyluric acids very effectively (Ashihara, et al. 1995). Absorbance at 270 and 290 nm was monitored using a Shimadzu type SPD-10A, UV-vis detector.

3.4. Metabolism of radiolabelled precursors

The metabolism of [methyl-^{14}C] SAM, [8-^{14}C]adenosine and [8-^{14}C]caffeine were investigated using procedures described by Koshiishi et al. (2001) and Ashihara et al. (1995). Bud segments or leaf discs (ca. 150 mg fresh weight) were incubated in 2 ml of medium, comprising 30 mM potassium phosphate buffer, pH 5.6, 10 mM sucrose and 37 kBq of ^{14}C-labelled substrate. Specific activity of labelled compounds and incubation condition of each experiment are shown in the table legends. Analysis of radiolabelled compounds by TLC and HPLC was as described by Ashihara et al. (1996) except that an additional solvent (EtoAc–MeOH–H_2O (100:13.5:10, v/v) was used for TLC, and autoradiography was conducted using an Image-Analyser system (FLA-2000, Fuji Photo Film Co., Ltd. Tokyo, Japan) and HPLC-radiocounting used a Ramona 2000 radioactivity monitor (Reytest Isotopenmessgerate Gmbh, Straubenhardt, Germany).

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