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Poppy alkaloid profiling by electrospray tandem mass spectrometry and electrospray FT-ICR mass spectrometry after [*ring*-$^{13}C_6$]-tyramine feeding

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Abstract

Papaver alkaloids play a major role in medicine and pharmacy. In this study, [*ring*-$^{13}C_6$]-tyramine as a biogenetic precursor of these alkaloids was fed to *Papaver somniferum* seedlings. The alkaloid pattern was elucidated both by direct infusion high-resolution ESI-FT-ICR mass spectrometry and liquid chromatography/electrospray tandem mass spectrometry. Thus, based on this procedure, the structure of about 20 alkaloids displaying an incorporation of the labeled tyramine could be elucidated. These alkaloids belong to different classes, e.g. morphinan, benzylisoquinoline, protoberberine, benzo[*c*]phenanthridine, phthalide isoquinoline and protopine. The valuable information gained from the alkaloid profile demonstrates that the combination of these two spectrometric methods represents a powerful tool for evaluating biochemical pathways and facilitates the study of the flux of distant precursors into these natural products.

Keywords: *Papaver somniferum*; Electrospray; MS/MS; Poppy alkaloids; ^{13}C-Labelling; FT-ICR mass spectrometry

1. Introduction

The isoquinoline alkaloids represent a manifold class of compounds within the plant kingdom. Morphine, a complex natural product with wide legal and illegal use as an analgesic, is produced exclusively in the opium poppy *Papaver somniferum* L. together with other pharmaceutically desired tetrahydrobenzylisoquinoline-derived alkaloids, such as codeine, noscapine, papaverine and thebaine (Duke, 1985). The tetrahydrobenzylisoquinoline skeleton in plants is biosynthetically formed by condensation of dopamine and 4-hydroxyphenylacetaldehyde, both derived from (*S*)-tyrosine, to form the first alkaloid in the pathway, the trioxygenated intermediate (*S*)-norcoclaurine. (*S*)-Norcoclaurine is transformed to (*S*)-reticuline by methyltransfer and hydroxylation. (*S*)-Reticuline is a central intermediate in the biosynthetic pathway of *Papaver* alkaloids. It can undergo various intramolecular coupling reactions that lead to a plethora of alkaloid types, such as the morphinan, protoberberine, benzo[*c*]phenanthridine, phthalideisoquinoline and protopine, which are found mainly in species of the Papaveraceae, Monimiaceae, Ranunculaceae, Berberidaceae and Menispermaceae. (*S*)-Reticuline is clearly one of the most versatile molecules in plant secondary metabolism.

Abbreviations: ESI, electrospray ionization; CID, collision induced dissociation; LC–MS/MS, liquid chromatography–mass spectrometry/mass spectrometry; FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; SRM, selected reaction monitoring.

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While the biosynthetic pathway leading to the pentacyclic morphinan alkaloids has been largely elucidated (see Kutchan, 1998), the flux of carbon derived from the primary metabolite (*S*)-tyrosine into the multitude of alkaloid types has not been investigated in the opium poppy. As an alkaloid precursor, ^{13}C-labeled (*S*)-tyrosine is diluted during feeding experiments, such that the isotopic enrichment does not always allow for clear determination of the incorporation pattern. In comparison, [1-$^{13}C_6$]-tyramine, which is also a distant, but more specific, precursor to the alkaloids, is well incorporated into the target molecules as determined by ^{13}C NMR spectroscopy (Roberts et al., 1987). However, in order to detect labeled intermediates and end products of established and predicted biosynthetic pathways in low concentrations, mass spectrometry is the favored analytical tool of choice. Mass spectrometry coupled with the use of biosynthetic precursors labeled with stable isotopes allows that the labeled atoms be tracked during the course of biosynthesis. Recently, stable isotopes and tandem mass spectrometry were used for profiling the metabolism of glycerophospholipid species (''metabolipidomics'', Bleijerveld et al., 2006).

Electrospray mass spectrometry represents a very sensitive method for investigating alkaloids because of their good ion efficiencies. During the last decade, electrospray tandem mass spectrometry has been successfully applied in the analysis and characterization of isoquinoline-type alkaloids (Henion et al., 1994; Aebi and Henion, 1996; Bringmann et al., 1998; Fabre et al., 2000; Gioacchini et al., 2000; Raith et al., 2003; Stévigny et al., 2004; Wu and Moyer, 2004; Schmidt et al., 2005; Sturm et al., 2006; Wickens et al., 2006). On the other hand, the Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) represents a mass spectrometric method yielding an excellent resolving power, mass accuracy and sensitivity. During the last years, there have been first efforts to apply this method in analytical problems on metabolomics (Aharoni et al., 2002; Brown et al., 2005).

We have carried out an investigation using [*ring*-$^{13}C_6$]-tyramine as a biogenetic precursor of these alkaloids for feeding to *P. somniferum* seedlings. Feeding of [*ring*-$^{13}C_6$]-tyramine to germinating seeds of *P. somniferum* for a period of nine days in the light resulted in a high incorporation rate of label into secondary metabolites (up to ca. 50%). Using high-resolution electrospray (ESI) FT-ICR mass spectrometry and liquid chromatography/electrospray tandem mass spectrometry the course of the labeled precursor could be followed very effectively. The so obtained mass spectrometric data enabled the identification not only of all major alkaloids of interest, but also of minor and new, overlooked compounds occurring in trace amounts. Moreover, the investigation of the fragmentation behavior of labeled and unlabeled compounds yield a detailed insight in the nature of key ions. It is shown that the mass spectrometric methods used represent powerful metabolite profiling tools for evaluating the biochemical pathway leading from tyramine to benzylisoquinoline and morphinan alkaloids and other biogenically related compounds.

2. Results and discussion

2.1. High-resolution ESI-FT-ICR mass spectrometry

The alkaloid-containing, basidified fraction resulting from poppy seedling extraction was dissolved in methanol and investigated by direct-infusion ESI-FT-ICR mass spectrometry (Fig. 1a and 1b). The good ion efficiencies of alkaloids and a high isotopic enrichment of the metabolites allowed for an effective detection of a series of $[M+H]^+$ ions in the extract. The prominent *m*/*z* values of even-mass indicating nitrogen-containing compounds in the ESI-FT-ICR mass spectrum reflect a typical *Papaver* alkaloid pattern. Most of the alkaloids appeared in the mass range from *m*/*z* 300 to 400, as shown by the partial positive ion ESI-FT-ICR mass spectrum (Fig. 1b). The incorporation of [*ring*-$^{13}C_6$]-tyramine is indicated by a nominal mass shift of 6 amu. It is obvious that the main alkaloids with this mass shift have $[M+H]^+$ ions at *m*/*z* 312.15965 (thebaine, **18**) with the corresponding [*ring*-$^{13}C_6$]-labeled compound **18c** (*m*/*z* 318.17978), *m*/*z* 328.15466 (334.17490), *m*/*z* 330.17029 (336.19039), *m*/*z* 354.13394 (360.15415) and *m*/*z* 370.16534 (376.18531), respectively. As shown in Table 1, the ESI-FT-ICR mass spectrometric data are of high mass accuracy, the method is suited for detection of alkaloids. In most cases, the mass accuracies were in a range from 0.1 to ca. 1.5 ppm. The incorporation of $^{13}C_6$ is clearly evidenced by the FT-ICR mass spectrometric measurement.

In selected cases, the nominal mass can be resolved into two or three different *m*/*z* values representing unique elemental compositions. For example, *m*/*z* 332, which is occupied by three different masses, corresponds to the $^{13}C_6$-labeled cheilanthifoline (**10c**) at 332.15881, sanguinarine (**13**) at *m*/*z* 332.09237 and an unknown metabolite at *m*/*z* 332.17650, respectively (Table 1). The triplet at *m*/*z* 332 is well separated, the measured resolution of ca. 45.000 enables a good differentiation between potential overlapping ions. Furthermore, an estimation of the $^{13}C_6$-enrichment of the different masses can be accomplished with such measurements. Since different *Papaver* alkaloids can have the same elemental composition (e.g. *N*-methylcoclaurine (**6**) and codeine (**19**)), LC–MS/MS experiments are necessary to identify and characterize the various alkaloids.

2.2. Liquid chromatography/electrospray tandem mass spectrometry

The alkaloid extract from the poppy seedlings was subjected to liquid chromatography/electrospray tandem mass spectrometry (LC-ESI-MS/MS). The collision-induced dissociation (CID) mass spectra of the resolved alkaloids yielded information not only to the type of alkaloids, but

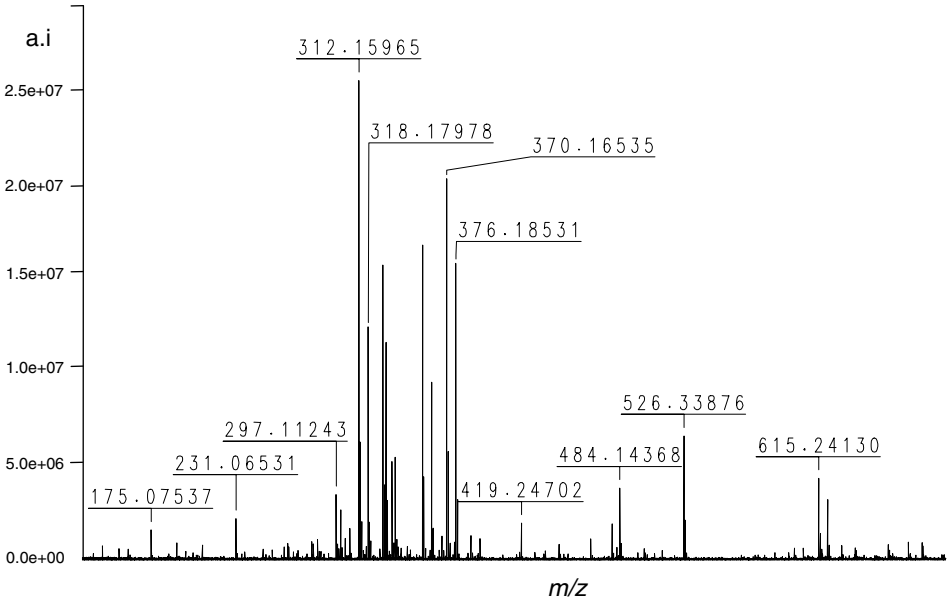


Fig. 1a. Positive ion ESI-FT-ICRMS of a poppy extract after feeding with [*ring*-$^{13}C_6$]-tyramine.

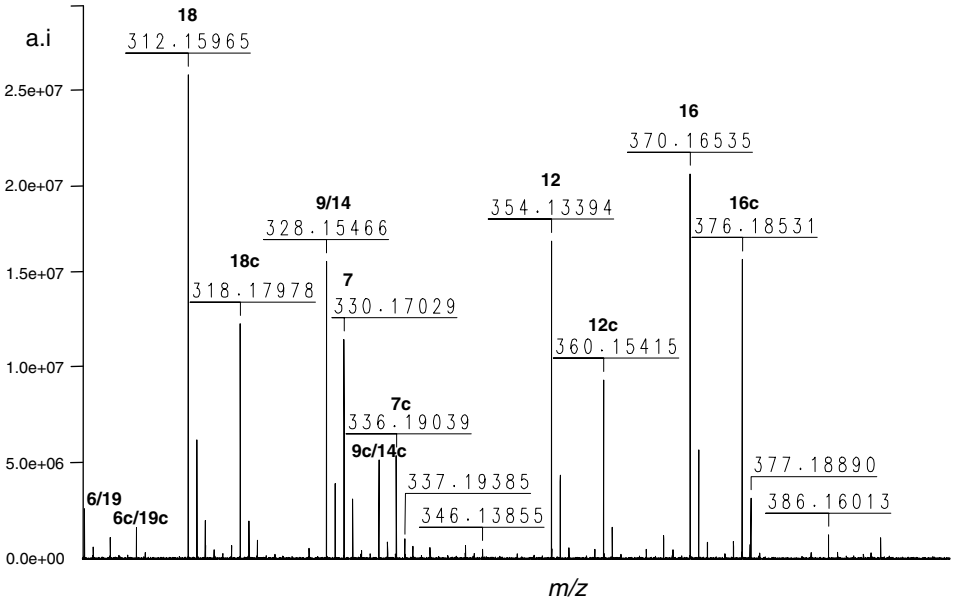


Fig. 1b. Partial positive ion ESI-FT-ICRMS of a poppy extract after feeding with [*ring*-$^{13}C_6$]-tyramine (the $^{13}C_6$-labeled compounds are designated with **c**).

also to the detailed structure of the alkaloid. The CID mass spectrum of the individual alkaloids was recorded along with that of the corresponding [*ring*-$^{13}C_6$]-labeled compounds. In most cases, reference compounds were available. Using tandem mass spectrometry, the identity of approximately 20 different alkaloids could be elucidated (Table 2). The biogenic relationship of the ^{13}C-enriched alkaloids is shown in Schemes 1 and 2.

The identity of the isoquinoline alkaloid salsolinol (**3**) formed by condensation of dopamine (**2**) and acetaldehyde was established by comparison with an authentic sample. The CID mass spectrum of the $[M+H]^+$ ion (*m*/*z* 180) showed a preferred loss of NH_3 leading to *m*/*z* 163, which is also the dominant product ion in the MS^2 spectrum obtained by ion trap MS (Song et al., 2006). Consecutive loss of CO forms the ion at *m*/*z* 145. In the case of the $^{13}C_6$-labeled compound **3c**, the corresponding key ions are shifted to *m*/*z* 169 and 151, respectively (Table 2).

The mass spectral behavior of the tetrahydrobenzylisoquinoline-type alkaloids (*S*)-norcoclaurine (**4**), (*S*)-coclaurine (**5**), (*S*)-*N*-methylcoclaurine (**6**) and (*S*)-reticuline (**7**) has been discussed previously (Schmidt et al., 2005). These compounds are well characterized; in the CID spectra of the corresponding [*ring*-$^{13}C_6$]-labeled compound, the frag-

Table 1
ESI-FT-ICR mass spectral data of the basidified fraction of extraction of poppy seedlings that were applied with [*ring*-$^{13}C_6$]-tyramine

Compound name(s)	No.	*m/z* ([M+H]$^+$), unlabeled	Relative intensity (%)	Elemental composition	Error (ppm)	*m/z* ([M+H]$^+$, $^{13}C_6$-labeled)	Relative intensity (%)	Elemental composition	Error (ppm)	$^{13}C_6$-Content (%)
Dopamine	**2**	154.08616	2.34	$^{12}C_8H_{12}NO_2$	0.6	160.10637	2.22	$^{12}C_2{}^{13}C_6H_{12}NO_2$	0.1	49
Salsolinol	**3**	180.10196	0.76	$^{12}C_{10}H_{14}NO_2$	0.3	186.12206	0.80	$^{12}C_4{}^{13}C_6H_{14}NO_2$	0.1	51
Norcoclaurine	**4**	272.12844	1.97	$^{12}C_{16}H_{18}NO_3$	1.2	278.14842	1.28	$^{12}C_{10}{}^{13}C_6H_{18}NO_3$	0.6	39
Not identified		272.11420	1.21	?		–		–		
Coclaurine/*N*-demethylcodeine	**5/20**	286.14406	1.79	$^{12}C_{17}H_{20}NO_3$	1.0	292.16422	1.35	$^{12}C_{11}{}^{13}C_6H_{20}NO_3$	1.1	43
N-Methylcoclaurine/codeine	**6/19**	300.15978	10.45	$^{12}C_{18}H_{22}NO_3$	1.2	306.17984	6.56	$^{12}C_{12}{}^{13}C_6H_{22}NO_3$	0.9	39
Thebaine	**18**	312.15965	100.00	$^{12}C_{19}H_{22}NO_3$	0.7	318.17978	48.58	$^{12}C_{13}{}^{13}C_6H_{22}NO_3$	0.7	37
Not identified		314.13905	8.02	$^{12}C_{18}H_{20}NO_4$	1.2	320.15922	3.93	$^{12}C_{12}{}^{13}C_6H_{20}NO_4$	1.3	33
Not identified		314.16660	2.90	?		–				
Codeine *N*-oxide	**21**	316.15391	1.17	$^{12}C_{18}H_{22}NO_4$	1.3	322.17507	1.00	$^{12}C_{12}{}^{13}C_6H_{22}NO_4$	1.9	46
Cheilanthifoline	**10**	326.13920	2.29	$^{12}C_{19}H_{20}NO_4$	1.6	332.15881	0.85	$^{12}C_{13}{}^{13}C_6H_{20}NO_4$	1.4	27
Scoulerine/corytuberine	**9/14**	328.15466	61.49	$^{12}C_{19}H_{22}NO_4$	1.0	334.17490	20.50	$^{12}C_{13}{}^{13}C_6H_{22}NO_4$	1.3	25
Reticuline	**7**	330.17029	45.35	$^{12}C_{19}H_{24}NO_4$	0.9	336.19039	21.40	$^{12}C_{13}{}^{13}C_6H_{24}NO_4$	0.8	32
Sanguinarine	**13**	332.09237	1.08	$^{12}C_{20}H_{14}NO_4$	1.9	338.11194	0.64	$^{12}C_{14}{}^{13}C_6H_{14}NO_4$	0.2	37
Not identified		332.17650	1.82	?		–				
N-Methylstylopine	**11**	338.13912	2.72	$^{12}C_{20}H_{20}NO_4$	1.3	344.15726	1.30	$^{12}C_{14}{}^{13}C_6H_{20}NO_4$	4.5	32
Not identified		340.11831	2.47	$^{12}C_{19}H_{18}NO_5$	1.1	346.13856	2.13	$^{12}C_{13}{}^{13}C_6H_{18}NO_5$	1.4	46
Papaverine ?		340.15440	1.35	$^{12}C_{20}H_{22}NO_4$	0.2	Not detectable				
Laudanine	**8**	344.18611	2.80	$^{12}C_{20}H_{26}NO_4$	1.4	350.20624	1.10	$^{12}C_{14}{}^{13}C_6H_{26}NO_4$	1.4	28
Protopine	**12**	354.13394	65.69	$^{12}C_{20}H_{20}NO_5$	1.0	360.15415	37.02	$^{12}C_{14}{}^{13}C_6H_{20}NO_5$	1.2	36
Not identified		368.09488	0.82	?		–				
Not identified		368.14966	1.37	$^{12}C_{21}H_{22}NO_5$	1.1	374.16992	1.14	$^{12}C_{15}{}^{13}C_6H_{22}NO_5$	1.4	45
Not identified		368.18639	2.01	$^{12}C_{22}H_{26}NO_4$	2.1	374.20629	0.94	$^{12}C_{16}{}^{13}C_6H_{26}NO_4$	1.4	32
Cryptopine	**16**	370.16534	79.51	$^{12}C_{21}H_{24}NO_5$	1.2	376.18531	61.90	$^{12}C_{15}{}^{13}C_6H_{24}NO_5$	0.7	44
Not identified		384.14471	1.39	$^{12}C_{21}H_{22}NO_6$	1.4	390.16477	1.02	$^{12}C_{15}{}^{13}C_6H_{22}NO_6$	1.2	42
Not identified		384.17998	1.17	$^{12}C_{22}H_{26}NO_5$	1.6	–				
Not identified	**17a/17b**	386.16013	5.02	$^{12}C_{21}H_{24}NO_6$	0.8	392.18036	4.47	$^{12}C_{15}{}^{13}C_6H_{24}NO_6$	1.1	47

Table 2
ESI-MS/MS data of *Papaver* alkaloids identified after feeding with [*ring*-$^{13}C_6$]-tyramine

Compound	Compound name	RT (min)	$[M+H]^+$ (*m/z*)	Ions obtained by ESI-CID (*m/z*, relative intensity)	CE (eV)	$^{13}C_6$-Content (%)
3	Salsolinol	5.7	180	180 (90), 163 ($[M+H-NH_3]^+$, 100), 151 (4), 145 ($[M+H-NH_3-CO]^+$, 41), 137 (11)	20	44
3c			186	186 (100), 169 ($[M+H-NH_3]^+$, 92), 157 (5), 151 (($[M+H-NH_3-CO]^+$, 40), 143 (5)	20	
4	Norcoclaurine	6.5	272	272 (15), 255 (38), 237 (8), 209 (4), 161 (**b**, 37), 145 (10), 143 (8), 123 (11), 107 (**c**, 100)	25	35
4c			278	278 (19), 261 (45), 243 (8), 214 (4), 167 (**b**, 36), 149 (8), 145 (9), 129 (10), 107 (**c**, 100)	25	
5	Coclaurine	7.8	286	286 (20), 269 (61), 254 (4), 237 (27), 209 (16), 175 (**b**, 34), 145 (11), 143 (8), 137 (11), 107 (**c**, 100)	25	36
5c			292	292 (22), 275 (59), 260 (7), 243 (29), 214 (12), 181 (**b**, 27), 149 (6), 145 (10), 143 (12), 107 (**c**, 100)	25	
6	*N*-Methylcoclaurine	7.1	300	300 (79), 269 (54), 237 (31), 209 (19), 175 (**b**, 43), 145 (18), 143 (15), 107 (**c**, 100)	25	35
6c			306	306 (67), 275 (59), 243 (38), 214 (22), 181 (**b**, 30), 145 (16), 143 (19), 107 (**c**, 100)	25	
7	Reticuline	10.0	330	330 (31), 299 (12), 267 (4), 239 (2), 192 (**a**, 100), 175 (**b**, 12), 143 (4), 137 (**c**, 11)	25	26
7c			336	336 (24), 305 (11), 273 (3), 244 (1), 198 (**a**, 100), 181 (**b**, 10), 149 (3), 137 (**c**, 7)	25	
8	Laudanine	8.9	344	344 → 206 (**a**, SRM)	25	22
8c			350	350 → 212 (**a**, SRM)	25	
9	Scoulerine	7.1	328	328 (33), 178 (**a**, 100), 151 (**d**, 18)	30	21
9c			334	334 (36), 184 (**a**, 100), 157 ($\mathbf{d}_1$, 14), 151 ($\mathbf{d}_2$, 6)	30	
10	Cheilanthifoline	[a]	326	326 → 178 (**a**, SRM)	30	16
10c			332	332 → 184 (**a**, SRM)	30	
11	*N*-Methylstylopine	31.5	338	338 (12), 190 (**a**, 100), 188 (4)	35	25
11c			344	344 (14), 196 (**a**, 100), 194 (3)	35	
12	Protopine	22.5	354	354 (100), 336 (8), 275 (19), 247 (9), 206 ($\mathbf{a}_1$, 22), 189 ($\mathbf{a}_2$, 70), 188 (($\mathbf{a}_2$-H), 65), 165 (**g**, 11), 149 (**e**, 31)	30	20
12c			360	360 (100), 342 (11), 281 (15), 253 (8), 212 ($\mathbf{a}_1$, 16), 195 ($\mathbf{a}_2$, 59), 194 (($\mathbf{a}_2$-H), 46), 165 (**g**, 8), 155 (**e**, 22)	30	
13	Sanguinarine	[a]	332	332 → 274 ($[M+H-CH_2O-CO]^+$, SRM)	35	26
13c			338	338 → 280 ($[M+H-CH_2O-CO]^+$, SRM)	35	
14	Corytuberine	5.2	328	328 (5), 297 77), 282 (63), 265 (100), 237 (34), 222 (11), 219 (16), 209 (8), 191 (9)	30	23
14c			334	334 (4), 303 (59), 288 (42), 271 (100), 243 (24), 228 (5), 225 (8), 214 (3), 197 (5)	30	
15	Berberine	[a]	336	336 → 320 (SRM)	40	23
15c			342	342 → 326 (SRM)	40	
16	Cryptopine	22.2	370	370 (44), 263 (15), 222 ($\mathbf{a}_1$, 52), 205 ($\mathbf{a}_2$, 100), 204 (($\mathbf{a}_2$-H), 82), 190 (**f**, 63), 175 (31), 165 (**e**, 52)	35	32
16c			376	376 (35), 269 (23), 228 ($\mathbf{a}_1$, 36), 211 ($\mathbf{a}_2$, 58), 210 (($\mathbf{a}_2$-H), 100), 196 (**f**, 34), 181 (9), 171 (**e**, 13)	35	
17a	Papaverrubine type	14.8	386	386 (29), 368 (100), 297 (29), 266 (7), 206 (**a**, 33), 165 (4), 44 (4)	30	56
17ac	X1		392	392 (44), 374 (100), 303 (38), 272 (7), 212 (**a**, 43), 171 (4), 44 (6)	30	
17b	Papaverrubine type	17.1	386	386 (48), 368 (20), 206 (**a**, 100)	30	34
17bc	X2		392	392 (89), 374 (34), 212 (**a**, 100)	30	

(*continued on next page*)

Table 2 (*continued*)

Compound	Compound name	RT (min)	$[M+H]^+$ (*m/z*)	Ions obtained by ESI-CID (*m/z*, relative intensity)	CE (eV)	$^{13}C_6$-Content (%)
18	Thebaine	16.0	312	312 (23), 281 (5), 266 (8), 255 (2), 249 (8), 221 (2), 58 (**h**, 100)	20	29
18c			318	318 (25), 287 (5), 272 (8), 261 (2), 255 (8), 227 (1), 58 (**h**, 100)	20	
19	Codeine[b]	6.2	300	300 (62), 225 (39), 215 (51), 199 (82), 193 (38), 183 (47), 171 (54), 165 (100), 58 (**h**, 75), 44 (**i**, 55)	38	37
19c			306	306 (100), 231 (31), 220 (47), 203 (60), 199 (19), 188 (51), 174 (26), 170 (47), 58 (**h**, 59), 44 (**i**, 58)	38	
20	*N*-Desmethylcodeine	9.3	286	286 (77), 225 (35), 209 (30), 191 (40), 181 (46), 165 (64), 153 (27), 121 (27), 91 (19), 44 (**h**, 100)	38	20
20c			292	292 (46), 231 (50), 203 (82), 197 (48), 187 (25), 170 (52), 159 (23), 127 (20), 96 (20), 44 (**h**, 100)	38	
21	Codeine-*N*-oxide	9.5	316	316 (50), 299 (71), 298 (37), 280 (54), 254 (17), 239 (25), 229 (46), 162 (100), 74 (**h**, 13), 60 (**i**, 22)	35	16
21c			322	322 (64), 305 (51), 304 (28), 288 (56), 259 (20), 243 (29), 231 (49), 168 (100), 74 (**h**, 20), 60 (**i**, 24)	35	

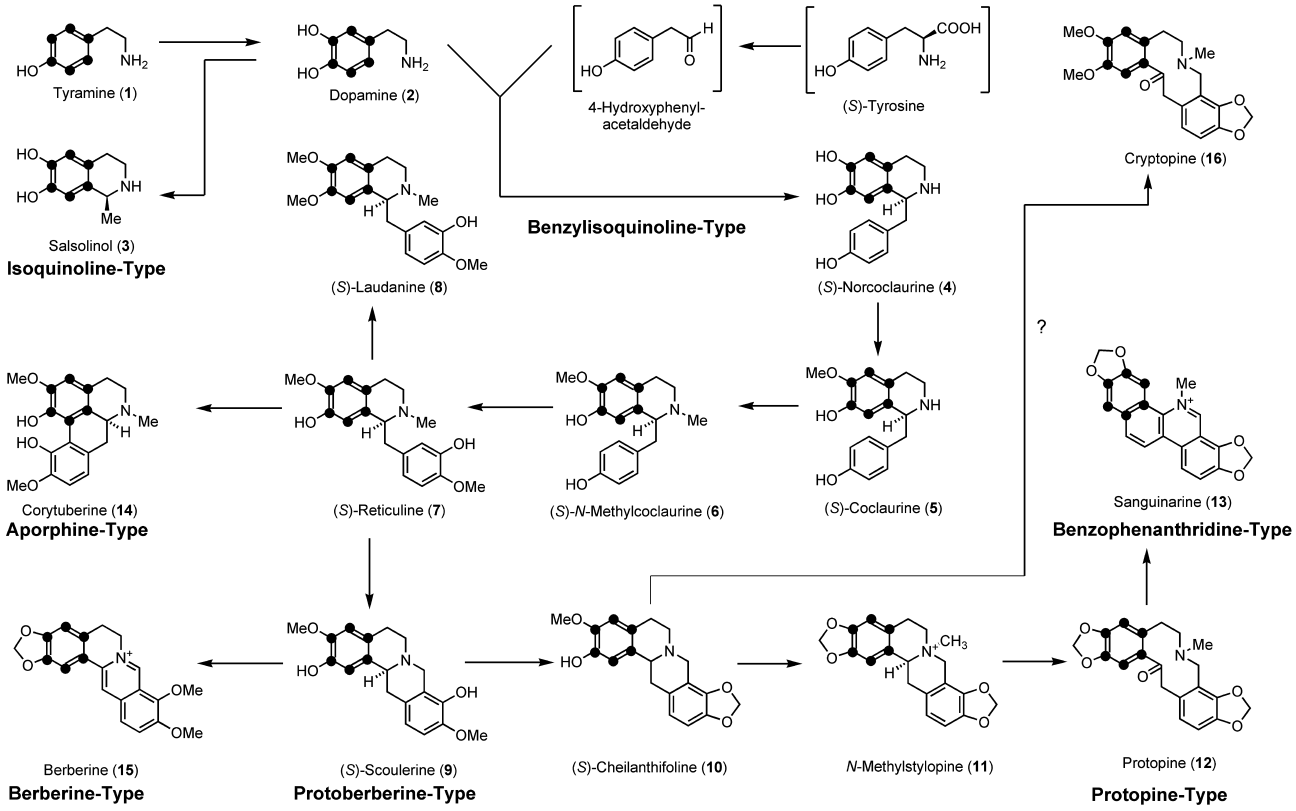
[a] These compounds were determined by LC-ESI-SRM using a Quantum Ultra AM.
[b] From Poeaknapo et al. (2004); CE = collision energy.

ment ions possessing the tyramine moiety show a mass shift of 6 amu (Fig. 2a and 2b). While in case of the coclaurine-type alkaloids **4**–**6** without an oxygen function in 3′O-position, the ion **c** comprising the benzyl moiety (**4**: *m/z* 107) becomes the base peak, the fragment ion **b** at *m/z* 161 in **4** is formed from the $[M+H-NH_3]^+$ ion (Fig. 2a and 2b, Scheme 3). In the course of this fragmentation, a rearrangement takes place forming a stable naphthalene-derived ion. If an oxygenated substituent is present in the 3′-position, the **a**-type ion (benzylisoquinoline moiety) will be the base peak as in case of reticuline (**7**: *m/z* 192) and laudanine (**8**: *m/z* 206, see Table 2).

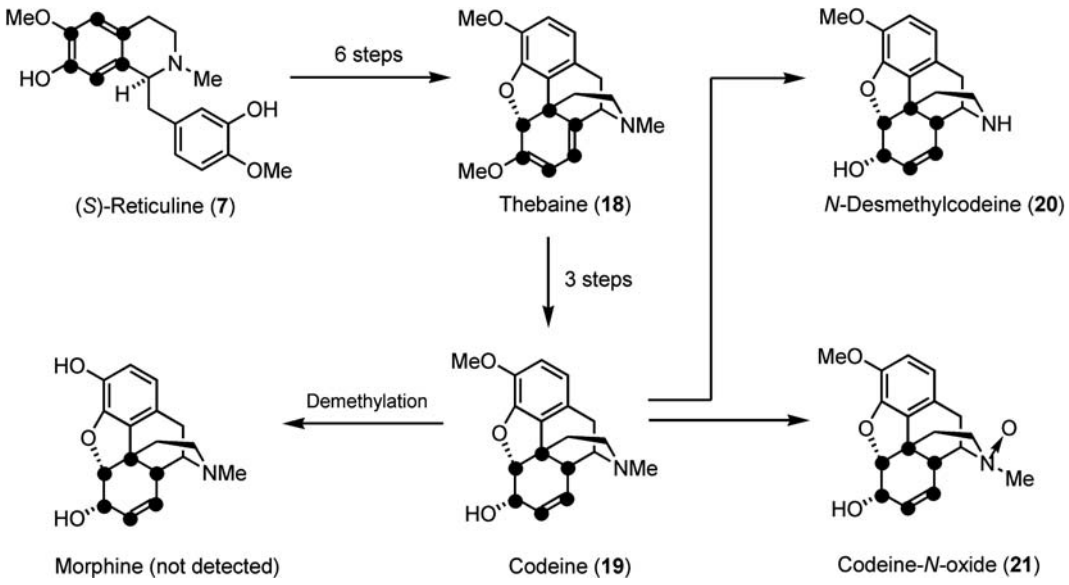
It is noteworthy that the benzyl moiety of norcoclaurine (fragment *m/z* 107, Fig. 2a and 2b) is, in the case of [*ring*-$^{13}C_6$]-tyramine feeding, devoid of label while the isoquinoline moiety carries the [*ring*-$^{13}C_6$]-label from tyramine. If the [*ring*-$^{13}C_6$]-tyramine administered to poppy seedling would be transformed by action of a monoamine oxidase to 4-hydroxyphenylacetaldehyde, a substrate of (*S*)-norcoclaurine synthase (Schumacher et al., 1983; Samanani and Facchini, 2001), both aromatic rings of norcoclaurine should contain ^{13}C label. This demonstrates that the reaction leading to the aldehydic substrate does not proceed via oxidative deamination of tyramine, but rather by decarboxylation of 4-hydroxyphenylpyruvate (Rueffer and Zenk, 1987). This same asymmetric labeling pattern is observed in protopine (Fig. 3b), which was derived from the [*ring*-$^{13}C_6$]-tyramine feeding experiment. Monoamine oxidase does not appear to have a role in the biosynthesis of alkaloids in poppy.

(*S*)-Reticuline represents a key compound in the biosynthesis of *Papaver* alkaloids forming in a first step both the protoberberine-type alkaloid scoulerine (**9**) and the aporphine corytuberine (**14**) as evidenced by the labeling experiment (Scheme 1). The LC/ESI-MS/MS analysis clearly showed that the $[M+H]^+$ ion at *m/z* 328 ($C_{19}H_{22}NO_4$, see Table 1) consists of these two alkaloids displaying quite a different fragmentation pattern (Scheme 4, Table 2). The CID mass spectrum of scoulerine is characterized by the key ions of type **a** comprising the isoquinoline moiety and an ion at *m/z* 151 (**d**-type) represents the complementary part (Frick et al., 2005). However, according to the CID mass spectrum of the $^{13}C_6$-labeled compound **9c** the ion at *m/z* 151 stems both from the isoquinoline moiety as shown by a mass shift to *m/z* 157 ($\mathbf{d}_1$) and the benzylic part which fragment ($\mathbf{d}_2$) is not shifted in **9c**. The second compound with a $[M+H]^+$ ion at *m/z* 328 belongs to the aporphine type. The CIDMS data are in agreement with those obtained from corytuberine (**14**, Scheme 4, Table 2). Prominent key ions of type $[M+H-MeNH_2]^+$ and $[M+H-MeNH_2-MeOH]^+$ were also observed in the MS^2 spectra of aporphine alkaloids obtained by an ion trap instrument (Stévigny et al., 2004). The ratio corytuberine/scoulerine was ca. 8:1 according to the LC–MS/MS data.

Traces of berberine (**15**), which was not detectable in the FT-ICR experiment, could be found by LC/ESI-



Scheme 1. Biosynthesis of selected *Papaver* alkaloids detected after feeding with [*ring*-$^{13}C_6$]-tyramine.



Scheme 2. Morphinan alkaloids detected after feeding with [*ring*-$^{13}C_6$]-tyramine.

SRM. In that case, the selected reaction monitoring mode leading to the base peak ion (*m*/*z* 336/342 → *m*/*z* 320/326, $[M+H-CH_4]^+$) was used for detection of this alkaloid that is not commonly found in opium poppy (Table 2).

Starting from scoulerine (**9**), the protoberberine alkaloids cheilanthifoline (**10**) and *N*-methylstylopine (**11**) are formed (Scheme 1). The occurrence of cheilanthifoline and its labeled derivative **10c** was determined by LC-ESI-SRM. In analogy to scoulerine, the formation of the isoquinoline fragment **a** (*m*/*z* 178/184) was used for the determination of cheilanthifoline. The same method was used to ascertain the presence of the benzophenanthridine-type alkaloid sanguinarine (**13**) ($[M+H]^+$ ion at *m*/*z* 332/338) with detection of the key ion at *m*/*z* 274/280

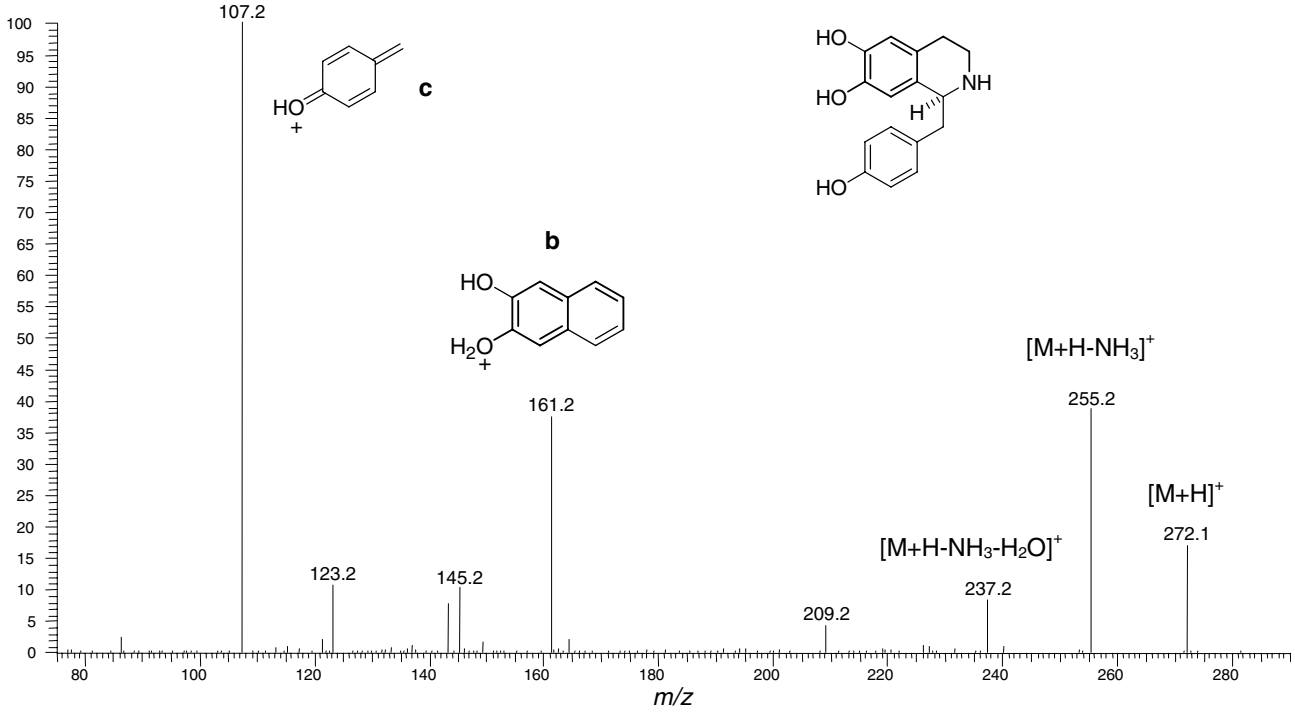


Fig. 2a. 25 eV positive ion ESI-CID mass spectrum of norcoclaurine (**4**).

([M+H−CH_2O−CO]$^+$). Full scan ESI-CID data of **13** were published elsewhere (Frick et al., 2005). Both cheilanthifoline (**10**) and sanguinarine (**13**) could be detected in only trace amounts.

As one can see in the ESI-FT-ICR mass spectrum (Fig. 1b), besides thebaine (**18**) the two protopine-type alkaloids protopine (**12**) and cryptopine (**16**) represent main compounds possessing the [*ring*-$^{13}C_6$]-label (Table 1). In general, the fragmentation behavior of **12** and **16** shows similarities to that of the benzylisoquinoline alkaloids. The key ion of type $\mathbf{a}_1$ corresponds to the typical even-numbered fragment comprising the isoquinoline moi-

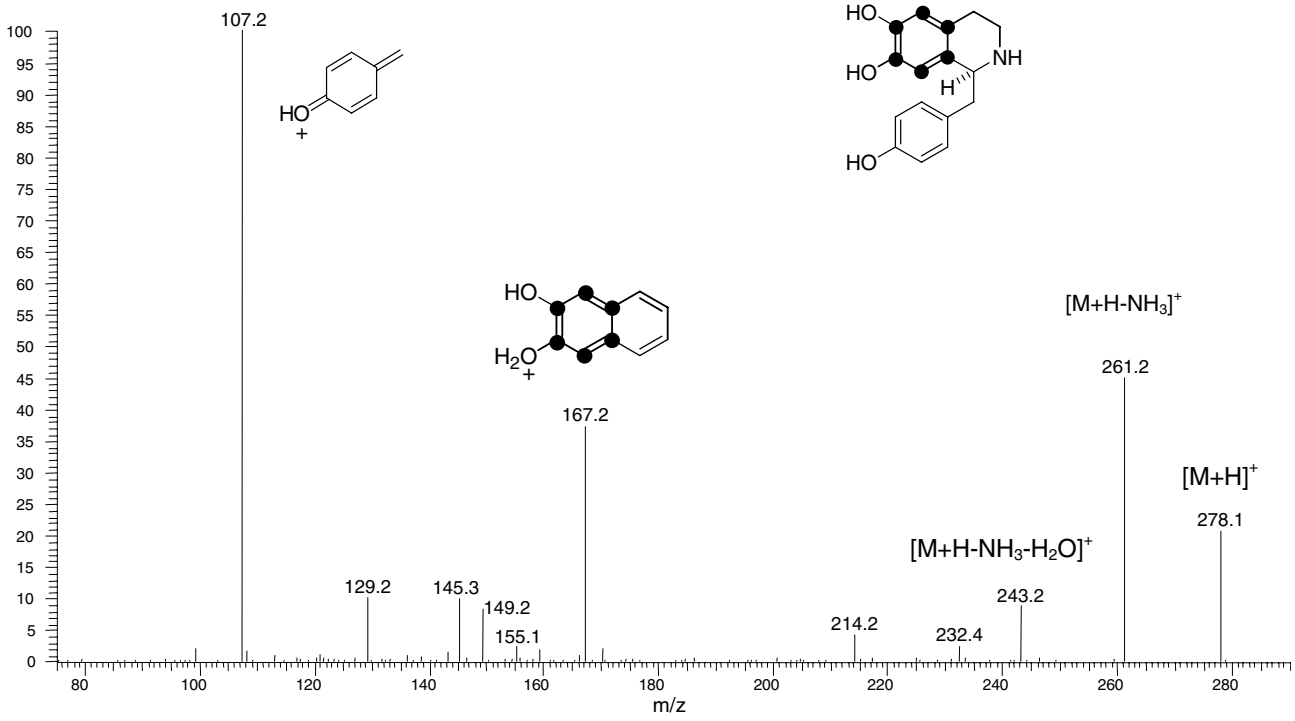
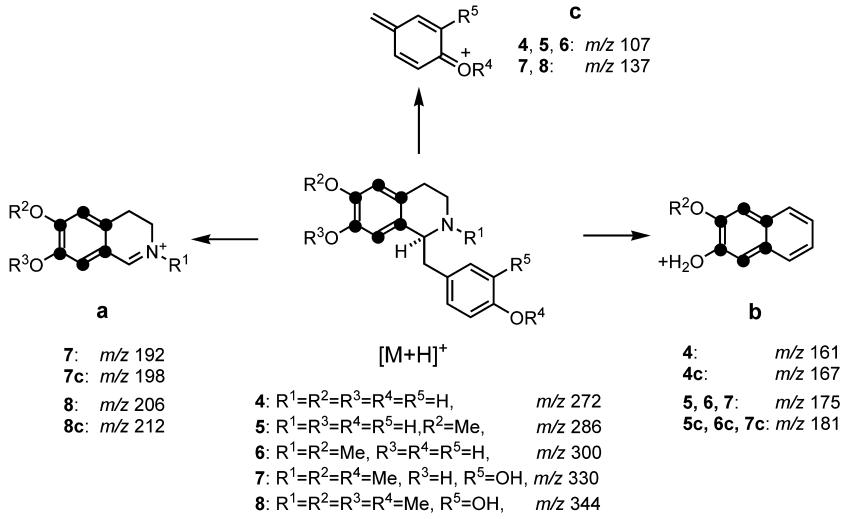
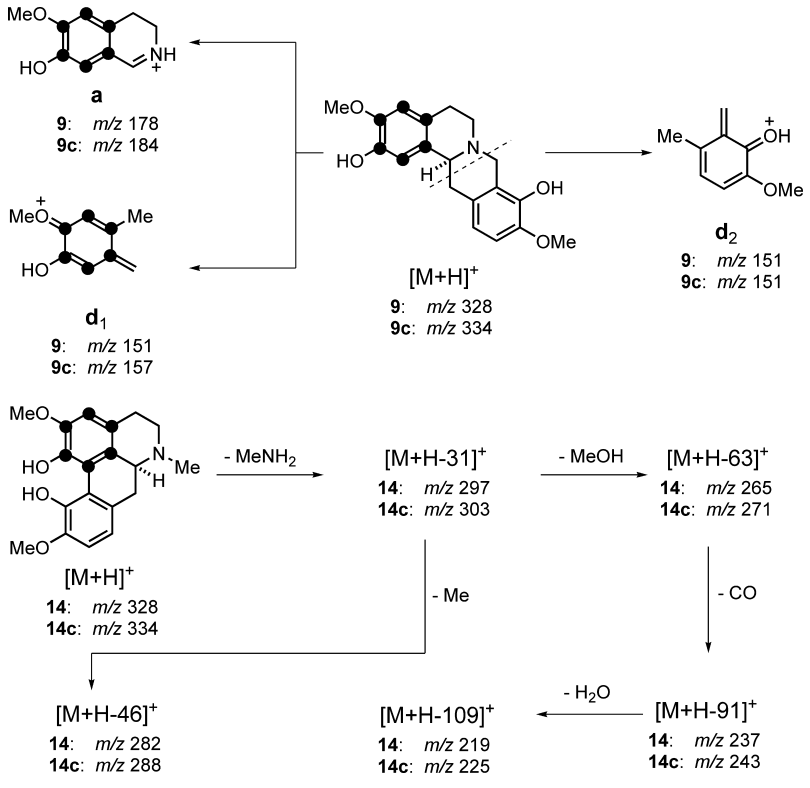


Fig. 2b. 25 eV positive ion ESI-CID mass spectrum of $^{13}C_6$-norcoclaurine (**4c**) after feeding with [*ring*-$^{13}C_6$]-tyramine.



Scheme 3. Key ions obtained in the ESI CID mass spectra of benzylisoquinoline alkaloids and their $^{13}C_6$-labeled derivatives.



Scheme 4. Mass spectral fragmentation of scoulerine (**9**) and corytuberine (**14**) as well as their $^{13}C_6$-labeled derivatives under electrospray CID conditions.

ety and the oxygen at C-1 (Fig. 3a, Scheme 5). This ion can loose water to form the ions of type ($\mathbf{a}_2$-H) at *m/z* 188 (**12**) and 204 (**16**). However, an abundant odd-numbered fragment $\mathbf{a}_2$ (**12**: *m/z* 189, **16**: *m/z* 205) appears also shifted in the CID mass spectra of **12c** and **16c** (Fig. 3b, Table 2). As evidenced by high-resolution mass measurements (see Section 4), protopine displays further significant fragments of type **e** and **g**. Ion **f** is only appearing in cryptopine. All relevant ions show the corresponding mass shift in the [*ring*-$^{13}C_6$]-labeled compounds (Scheme 5).

The $[M+H]^+$ ion at *m/z* 386 possessing an elemental composition of $C_{21}H_{24}NO_6$ belongs to two different alkaloids as shown by the LC–MS/MS experiment (Tables 1 and 2). Each alkaloid, detected only in trace amounts, showed incorporation of the [*ring*-$^{13}C_6$]-tyramine, but neither could be identified. These compounds (**17a** and **17b**)

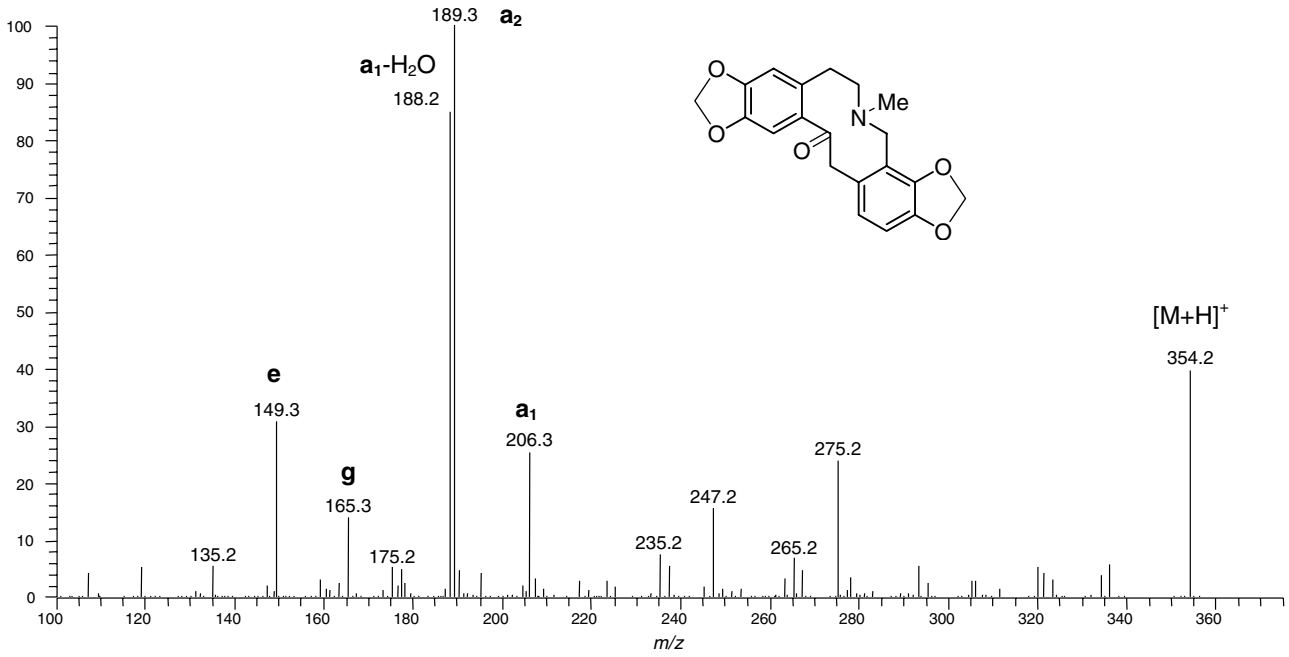
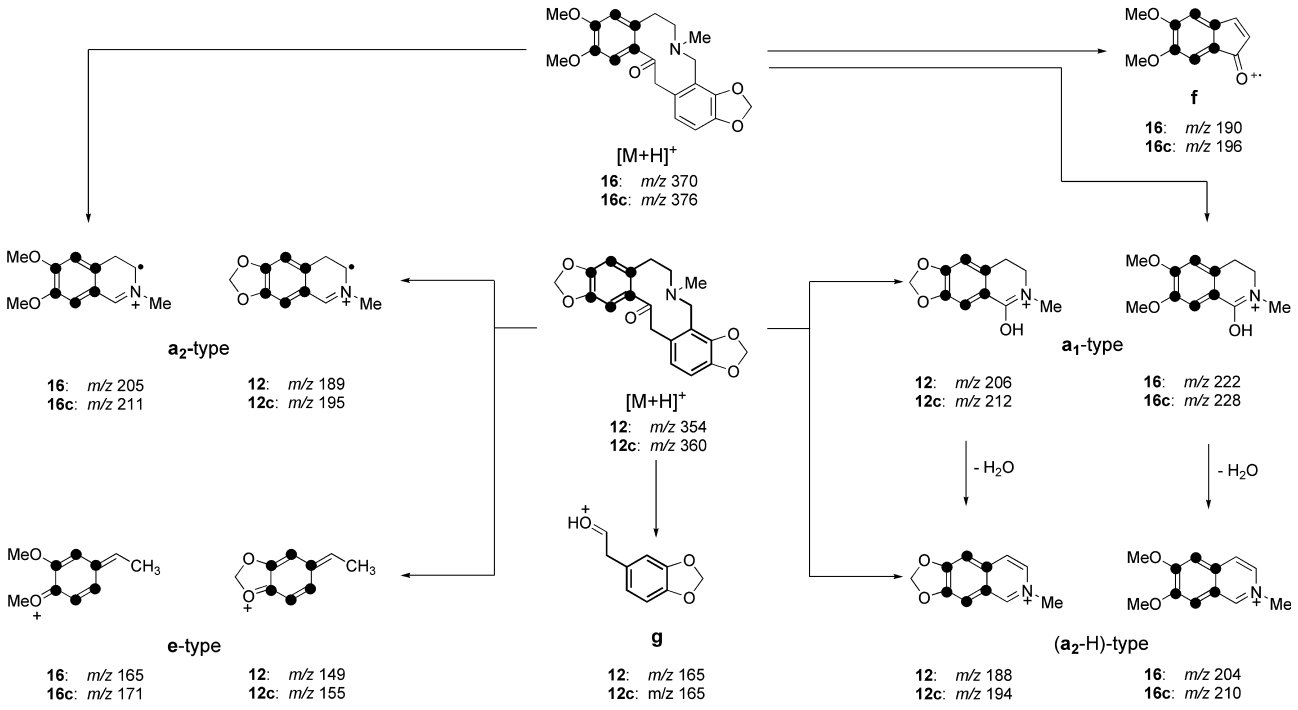


Fig. 3a. 30 eV positive ion ESI-CID mass spectrum of protopine (**12**).



Scheme 5. Mass spectral fragmentation of protopine (**12**) and cryptopine (**16**) as well as their $^{13}C_6$-labeled derivatives under electrospray CID conditions.

showing an intense ion **a** at *m*/*z* 206 can tentatively be assigned to the papaverrubine type.

The most abundant alkaloid detected, thebaine (**18**), belongs to the morphinan-type alkaloids. Its biosynthesis branches at (*S*)-reticuline (**7**) with an inversion of stereochemistry at C-1 to (*R*)-reticuline (Scheme 2). The epimers cannot be differentiated by LC–MS/MS. In addition to thebaine (**18**), the morphinans codeine (**19**), *N*-desmethylcodeine (**20**) and codeine-*N*-oxide (**21**) could be detected in the poppy seedling extract. The mass spectral behavior of thebaine and codeine under CID conditions was previously discussed in detail (Raith et al., 2003; Poeaknapo et al., 2004). The mass spectral behavior of *N*-desmethylcodeine (**20**) is very similar to that of codeine. The base peak at

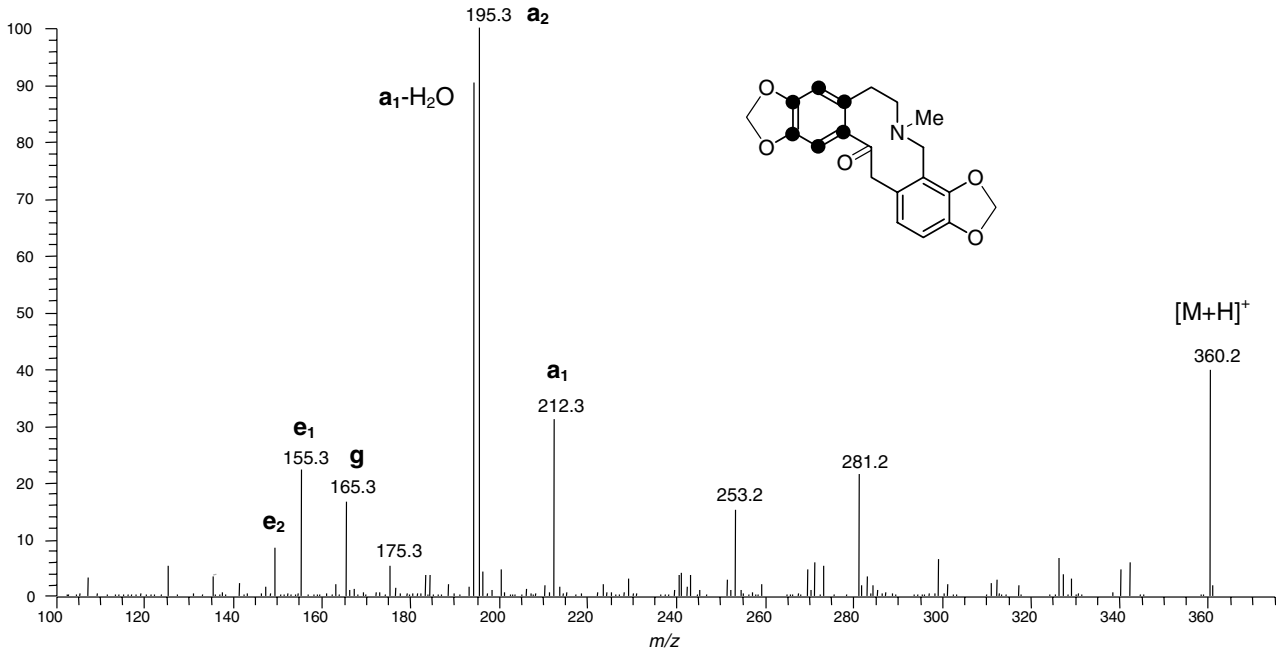
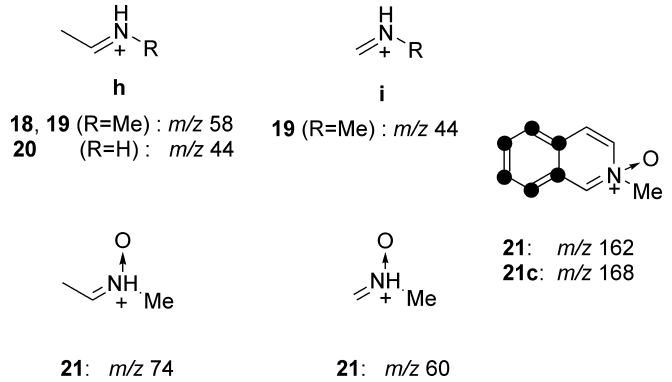


Fig. 3b. 30 eV positive ion ESI-CID mass spectrum of $^{13}C_6$-protopine (**12c**) after feeding with [*ring*-$^{13}C_6$]-tyramine.

m/*z* 44 (**h**) indicates the absence of the *N*-methyl group. In the CID mass spectra of codeine and thebaine possessing a *N*-methyl function, the corresponding ethylidene-ammonium type ion **h** appears at *m*/*z* 58 (Scheme 6, Table 2). Codeine-*N*-oxide (**21**), which shows a fragmentation pattern different from that of codeine, could be identified compared with authentic sample. The *N*-oxide function has a remarkable influence on the fragmentation behavior of this alkaloid. Thus, compound **21** displays a base peak at *m*/*z* 162 that is shifted to *m*/*z* 168 in the [*ring*-$^{13}C_6$]-labeled derivative **21c**, which comprises the isoquinoline moiety (Scheme 6, Table 2). In this case, the key ions of type **h** and **i** appear with the corresponding mass shift of 16 amu at *m*/*z* 74 and 60, respectively.



Scheme 6. The key ions **h**, **i** and *m*/*z* 162/168 in the morphinan alkaloids **18**–**21**.

3. Conclusions

The combined application of ^{13}C-labeled isotopic precursors to seedlings, direct infusion FT-ICR mass spectrometry and tandem mass spectrometry yield a very useful tool for investigating *Papaver* alkaloid metabolism. The high resolving power and mass accuracy of FT-ICR-MS analysis leads not only to a fast determination of the elemental composition of the detected alkaloids, but also to an estimation of the ^{13}C-content as well as the differentiation of isobaric ions (e.g. ^{13}C-labeled cheilanthifoline and non-labeled sanguinarine, both at *m*/*z* 332). It could be shown that even alkaloids occurring in trace amounts can be detected. Furthermore, there was also indication of the presence of nitrogen-free metabolites with an incorporation of $^{13}C_6$ upon thorough inspection of the FT-ICR MS (data not shown). For example, the peaks at *m*/*z* 255.10174 (calculated for $^{12}C_{16}H_{15}O_3$ 255.10157, 0.7 ppm) and *m*/*z* 261.12166 (calculated for $^{12}C_{10}{}^{13}C_6H_{15}O_3$ 261.12170, 0.2 ppm) hint at the presence of phenanthrene-derivative thebaol ($[M+H]^+$, *m*/*z* 255), a potential catabolite of thebaine. In a complementary analysis, LC–MS/MS investigations provided information for a structural characterization of the several alkaloids. The large diversity of alkaloids in opium poppy make an exhaustive structural elucidation a formidable task. In cases where one cannot determine the alkaloid structure by comparing with reference compounds or reference MS/MS data, the CID mass spectra can often yield clues to the structural classification of the alkaloids. Herein also lies a great

potential for ion trap mass spectrometry, especially using automated MS^2/MS^3 measurements, for such investigations.

Papaver somniferum serves as the sole source of several of the most important medically used plant secondary products. Three alkaloids are directly used in therapy – morphine (analgesic), codeine and noscapine (narcotine) are antitussive. Thebaine is a biosynthetic intermediate of the morphine pathway; it serves as a synthon for a variety of pharmaceuticals such as the analgesic oxycodone and the opiate antagonists naloxone and naltrexone. Due to the pharmaceutical importance of the parent alkaloids in this plant, and of the plant itself as the commercial source of the alkaloids, considerable effort has been invested in understanding morphine biosynthesis at the enzyme and gene levels. Nearly all of the enzymes of morphine biosynthesis have been identified and characterized, as have 10 of the genes (Kutchan et al., 2006). Using these genes, the first reports of metabolic engineering of opium poppy have begun to appear (Frick et al., 2004, 2006; Allen et al., 2004; Larkin et al., 2006). As knowledge is gained on the nature of alkaloid biosynthetic genes, increasing effort is invested in the metabolic engineering of plants to achieve tailored metabolite profiles. Currently, efforts at plant metabolic engineering are hit-and-miss, at best. The goal of predictive metabolic engineering relies on a systematic analysis of the metabolic profile and gene expression effects of introducing a transgene. Relatively little is understood about the flux of intermediates through alkaloid biosynthetic pathway and how common intermediates flow into highly branched pathways. The opium poppy as experimental system is suited to developing methods for secondary metabolite flux analysis due to the volume of knowledge on alkaloid biosynthesis in this plant at the cell biological, enzymatic and molecular genetic levels (Kutchan et al., 2006). As transgenic opium poppies with interesting alkaloid profiles continue to become available, mass spectrometric techniques as described herein will become centrally important to a thorough analysis of the effects of a transgene on metabolism.

In this first attempt to determine the fate of a ^{13}C-labeled distant alkaloid precursor in seedlings of *P. somniferum*, the metabolite profile indicates that a considerable amount of the [*ring*-$^{13}C_6$]-tyramine is diverted from morphine biosynthesis. Although not all ^{13}C-enriched structures have yet been elucidated, it is clear that at least the biosynthetic pathways leading to salsolinol (**3**), laudanine (**8**), aporphines and protoberberines compete with the morphinan pathway for common precursors such as (*S*)-reticuline. Even with incomplete profiling, key reactions begin to emerge such as scoulerine formation (by action of the berberine bridge enzyme) and aporphine oxidative coupling (likely a cytochrome P-450-dependent oxidase). Taken together with knowledge of the enzymes, genes and cell-specific expression of a pathway, this type of information on carbon flow through alkaloid biosynthesis is critical to the design of predictive metabolic engineering experiments.

4. Experimental

4.1. Application experiment with [*ring*-$^{13}C_6$]-tyramine to poppy seedlings

Seeds of an inbred *P. somniferum* variety "Munich" were sown into round plastic boxes (69 × 40 mm) supported by one layer of filter paper. The isotopic labeled [*ring*-$^{13}C_6$]-tyramine was synthesized (Poeaknapo et al., 2004) and added in 2.9 mM concentration. The plastic boxes were closed and exposed to constant illumination at 2000 lux (warm white fluorescent lamps). The seedlings (35 g) were harvested after nine days, frozen in liquid nitrogen, powdered with a mortar and pestle and extracted with warm 80% ethanol (200 ml). The extract was filtered, reduced in volume in vacuo, taken up in 1 N HCl and washed 3 × with chloroform. The acidic layer was then adjusted to pH 9.3 with 2 N KOH and extracted with chloroform. Chloroform layers were combined and dried in vacuo. The residue was taken up in ca. 0.2 ml methanol, centrifuged and used for mass spectrometric experiments.

4.2. ESI-FT-ICR mass spectrometry

The high-resolution ESI mass measurements of the poppy extract after [*ring*-$^{13}C_6$]-tyramine feeding were performed on a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, USA) equipped with an Infinity™ cell, a 7.0 T superconducting magnet (Bruker, Karlsruhe, Germany), an RF-only hexapole ion guide and an external APOLLO electrospray ion source (Agilent, off axis spray, voltages: endplate, −3.700 V; capillary, −4.200 V; capillary exit, −100 V; skimmer 1, 15.0 V; skimmer 2, 6.0 V). Nitrogen was used as drying gas at 150 °C. The sample solutions were introduced continuously via a syringe pump with a flow rate of 120 μl h^{-1}. The data were acquired with 512k data points and zero filled to 2048k by averaging 32 scans. The measured mass range was *m*/*z* 100–1000.

The $^{13}C_6$-content of the various mass peaks was calculated from the abundances of the unlabeled and the corresponding $^{13}C_6$-labeled ions.

The elemental composition of the protopine fragment ions were determined by measuring a reference protopine sample using a capillary exit voltage of −225 V: *m*/*z* 354.13175 ($[M+H]^+$, calc. for $C_{20}H_{20}NO_5$ 354.13092), 336.12256 ($[M+H-H_2O]^+$, calc. for $C_{20}H_{18}NO_4$ 336.12303), 323.09153 ($[M+H-CH_3NH_2]^+$, calc. for $C_{19}H_{15}O_5$ 323.09140), 275.07058 ($[M+H-CH_3NH_2-CH_2(OH)_2]^+$, calc. for $C_{18}H_{11}O_3$ 275.07027), 247.07558 ($[M+H-CH_3NH_2-CH_2(OH)_2-CO]^+$, calc. for $C_{17}H_{11}O_2$ 247.07536), 206.08118 (**a**$_1$, calc. for $C_{11}H_{12}NO_3$ 206.081170), 189.07818 (**a**$_2$, calc. for $C_{11}H_{11}NO_2$

189.07843), 188.07054 ([$\mathbf{a}_1-H_2O$], calc. for $C_{11}H_{10}NO_2$ 188.07060), 165.05447 (**g**, calc. for $C_9H_9O_3$ 165.05462), 149.05954 (**e**, calc. for $C_9H_9O_2$ 149.05971).

4.3. ESI-tandem mass spectrometry (MS/MS)

The positive ion electrospray ionization (ESI) mass spectra were obtained with a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4.5 kV; heated capillary temperature 220 °C; sheath gas: nitrogen) coupled with a Surveyor MicroLC system equipped with an RP18-column (5 μm, 1 × 100 mm, SepServ, Berlin). A gradient system ranging from H_2O:CH_3CN 90:10 (each of them contained 0.2% HOAc) to 15:85 over 15 min, followed by isocratic elution with a 10:90 mixture of both solvents for 25 min, was used; flow rate 25 μl min^{-1}. The CID mass spectra during a HPLC run were recorded with collision energies indicated in Table 2. Argon was used as collision gas (collision pressure: 1.8×10^{-3} Torr).

The LC-selected reaction monitoring (SRM) measurements were performed on a Quantum Ultra AM instrument (ThermoFinnigan, electrospray voltage 3.0 kV; heated capillary temperature 220 °C; sheath gas: nitrogen) coupled to a Surveyor MicroLC system equipped with a RP18-column (5 μm, 1 × 100 mm, SepServ, Berlin). A gradient system ranging from H_2O:CH_3CN 85:15 (each of them contained 0.2% HOAc) to 10:90 over 15 min, followed by isocratic elution with a 10:90 mixture of both solvents for 15 min, was used; flow rate 50 μl min^{-1}; collision pressure of argon: 1.5×10^{-3} Torr. The following retention times (RTs) were obtained: cheilanthifoline (**10**), 8.9 min; sanguinarine (**13**), 22.1 min; berberine (**15**), 19.1 min.

The $^{13}C_6$-content of the various compounds was calculated from the corresponding peak areas derived from LC–MS/MS-measurements of the unlabeled and the $^{13}C_6$-labeled ion.

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