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# Planta medica

## Journal of Medicinal Plant Research

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Organ der  
Gesellschaft für  
Arzneipflanzen-  
forschung

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## Contents

Volume 49, 1983



Hippokrates

ISSN 0032-0943  
Hippokrates Verlag Stuttgart

## Contents 49, 1983

<i>Atta-ur-Rahman, Bashir, M.</i> : Isolation of New Alkaloids from <i>Catharanthus roseus</i> . . . . .	124	Cannabinoids in <i>Phelipaea ramosa</i> , a Parasite of <i>Cannabis sativa <td>250</td> </i>	250
<i>Atta-ur-Rahman, Nisa, M., Farhi, S.</i> : Isolation of Moenjodaramine from <i>Buxus papilosa</i> . . . . .	126	<i>Galun, E., Aviv, D., Dantes, A., Freeman, A.</i> : Biotransformation by Plant Cells Immobilized in Cross-Linked Polyacrylamide-Hydrazide. Monoterpene Reduction by Entrapped <i>Mentha Cells</i> . . . . .	9
<i>Balsevich, J., Kurz, W. G. W.</i> : The Role of 9- and/or 10-oxygenated Derivatives of Geraniol, Geranial, Nerol, and Neral in the Biosynthesis of Loganin and Ajmalicine . . . . .	79	<i>Ghosal, Sh., Singh, A. K., Biswas, K.</i> : New 6-Aryl-2-pyrones from <i>Gentiana pedicellata</i> . . . . .	240
<i>Bassleer, R., Marnette, J.-M., Wiliquet, Ph., De Pauw-Gillet, M.-Cl., Caprasse, M., Angenot, L.</i> : Etude complémentaire de la cytotoxicité de la mélinonine F, alcaloïde dérivé de la $\beta$ -carboline (Complementary Study of Cytotoxic Activity of Melinonine F) . . . . .	158	<i>Hafez, A., Adolf, W., Hecker, E.</i> : Active Principles of the Thymelaeaceae. III. Skin Irritant and Cocarcinogenic Factors from <i>Pimelea simplex</i> . . . . .	3
<i>Becker, H., Chavadej, S., Weberling, F.</i> : Valepotriates in <i>Valeriana thalictroides</i> . . . . .	64	<i>Ieven, M., van den Berghe, D. A., Vlietinck, A. J.</i> : Plant Antiviral Agents. IV. Influence of Lycorine on Growth Pattern of Three Animal Viruses . . . . .	109
<i>Becker, H., Herold, S.</i> : RP-8 als Hilfsphase zur Akkumulation von Valepotriaten aus Zellsuspensionskulturen von <i>Valeriana wallichii</i> (RP-8 Auxiliary Phase for the Accumulation of Valepotriates from Cell-Suspension-Culture of <i>Valeriana wallichii</i> ) . . . . .	191	<i>Ishiguro, K., Yamaki, M., Takagi, S.</i> : Studies on Iridoid-related Compounds; III: Gentiopical, the Aglucone of Gentiopicroside . . . . .	208
<i>Bounthanh, C., Richert, L., Beck, J. P., Haag-Berrurier, M., Anton, R.</i> : The Action of Valepotriates on the Synthesis of DNA and Proteins of Cultured Hepatoma Cells . . . . .	138	<i>Jakovlev, V., Isaac, O., Flaskamp, E.</i> : Pharmakologische Untersuchungen von Kamillen-Inhaltsstoffen, VI. Untersuchungen zur antiphlogistischen Wirkung von Chama-zulen und Matricin (Pharmacological Investigations with Compounds of Chamomile, VI. Investigations on the Antiphlogistic Effects of Chamazulene and Matricine) . . . . .	67
<i>Briançon-Scheid, F., Lobstein-Guth, A., Anton, R.</i> : HPLC Separation and Quantitative Determination of Biflavones in Leaves from <i>Ginkgo biloba</i> . . . . .	204	<i>Joshi, K. C., Singh, P., Taneja, S.</i> : A Sesquiterpenoid Naphthol from <i>Kydia calycina</i> . . . . .	127
<i>Caputo, O., Delprino, L., Viola, F., Caramiello, R., Baliano, G.</i> : Biosynthesis of Sterols and Triterpenoids in Tissue Cultures of <i>Cucurbita maxima</i> . . . . .	176	<i>Jossang, A., Lebœuf, M., Cabalion, P., Cavé, A.</i> : Alcaloïdes des Annonacées. XLV: Alcaloïdes de <i>Polyalthia nitidissima</i> (Alkaloids from Annonaceae. XLV: Alkaloids of <i>Polyalthia nitidissima</i> ) . . . . .	20
<i>Chagnon, M., Ndiwbami, A., Dubé, S., Bumaya, A.</i> : Activite Anti-Inflammatoire d'Extraits de <i>Crassocephalum multicorymbosum</i> (Anti-inflammatory Action of an Extract from <i>Crassocephalum multicorymbosum</i> ) . . . . .	255	<i>Kimura, Y., Ohminami, H., Okuda, H., Baba, K., Kozawa, M., Arichi, S.</i> : Effects of Stilbene Components of Roots of <i>Polygonum</i> ssp. on Liver Injury in Peroxidized Oil-fed Rats . . . . .	51
<i>Chatopadhyay, S., Chatopadhyay, U., Mathur, P. P., Saini, K. S., Ghosal, S.</i> : Effects of Hippadine, an Amaryllidaceae Alkaloid, on Testicular Function in Rats . . . . .	252	<i>Kisiel, W.</i> : 8-Epidesacylcynaropicrin from <i>Crepis capillaris</i> . . . . .	246
<i>Chen Weiming, Yan Yaping, Liang Xiaotian</i> : Alkaloids from Roots of <i>Alstonia yunnanensis</i> . . . . .	62	<i>Kiso, Y., Suzuki, Y., Watanabe, N., Oshima, Y., Hikino, H.</i> : Antihepatotoxic Principles of <i>Curcuma longa</i> Rhizomes . . . . .	185
<i>Dehaussy, H., Tits, M., Angenot, L.</i> : La guattégaumérine, nouvel alcaloïde bisbenzylisoquinoléinique de <i>Guatteria gaumeri</i> (Guattegaumerine, New Bisbenzylisoquinoline Alkaloid from <i>Guatteria gaumeri</i> ) . . . . .	25	<i>Kiso, Y., Tohkin, M., Hikino, H.</i> : Assay Method for Anti-hepatotoxic Activity Using Carbon Tetrachloride Induced Cytotoxicity in Primary Cultured Hepatocytes . . . . .	222
<i>Domínguez, X. A., Franco, R., Cano, G., Ma. Consue lo García, F., Xorge A. Domínguez, S. Jr., Leonardo de la Peña, M.</i> : Isolation of a New Furano-1,4-Naphthaquinone, Diodantunezone from <i>Lanthena achyranthifolia</i> . . . . .	63	<i>Kram, G., Franz, G.</i> : Untersuchungen über die Schleim-polysaccharide aus Lindenblüten (Analysis of Linden Flower Mucilage) . . . . .	149
<i>Endo, K., Oshima, Y., Kikuchi, H., Koshihara, Y., Hikino, H.</i> : Hypotensive Principles of <i>Uncaria Hooks</i> . . . . .	188	<i>Krüger, D., Junior, P., Wichtl, M.</i> : Neue Cardenolidglykoside aus <i>Digitalis lanata</i> (New Cardiac Glycosides from <i>Digitalis lanata</i> ) . . . . .	74
<i>Engelshowe, R.</i> : Dimere Proanthocyanidine als Gerbstoffvorstufen in <i>Juniperus communis</i> (Tannin Producing Dimeric Proanthocyanidins in <i>Juniperus communis</i> ) . . . . .	170	<i>Langhammer, L., Schulze, G., Gujer, R., Magnolato, D., Horisberger, M.</i> : Isolation and Structure of a Rarely Occurring Cyanidanol Glycoside from Cortex Betulae . . . . .	181
<i>Esterl, A., Gäb, S., Bieniek, D.</i> : Zur Kenntnis der Inhaltsstoffe von <i>Isertia hypoleuca</i> (On The Constituents of <i>Isertia hypoleuca</i> ) . . . . .	244	<i>Lemli, J., Cuveele, J., Verhaeren, E.</i> : Chemical Identification of Alexandrian and Tinnevely Senna. Studies in the Field of Drugs Containing Anthracene Derivatives XXXIV . . . . .	36
<i>Fournier, G., Paris, M.</i> : Mise en Evidence de Cannabinoïdes Chez <i>Phelipaea ramosa</i> , Orobanchacees, Parasitant le Chanvre, <i>Cannabis sativa</i> , Cannabinacees (Detection of		<i>Long-Ze Lin, Wagner, H., Seligmann, O.</i> : Thalifaberine, Thalifabine and Huangshanine, Three New Dimeric Aporphine-Benzylisoquinoline Alkaloids . . . . .	55

Man-Po Wong, Teh-Chang Chiang, Hson-Mou Chang: Chemical Studies on Dangshen, the Root of <i>Codonopsis pilosula</i> . . . . .	60	Rueffer, M., Nagakura, N., Zenk, M. H.: Partial Purification and Properties of S-Adenosylmethionine: (R), (S)-Norlaudanosoline-6-O-Methyltransferase from <i>Argemone platyceras</i> Cell Cultures . . . . .	131
Misawa, M., Hayashi, M., Takayama, S.: Production of Antineoplastic Agents by Plant Tissue Cultures. I. Induction of Callus Tissues and Detection of the Agents in Cultured Cells . . . . .	115	Sariyar, G.: Alkaloids from <i>Papaver triniifolium</i> of Turkish Origin . . . . .	43
Nahrstedt, A., Wray, V., Grotjahn, L., Fikenscher, L. H., Hegnauer, R.: New Acylated Cyanogenic Diglycosides from Fruits of <i>Anthemis cairica</i> . . . . .	143	Seip, E. H., Ott, H. H., Hecker, E.: Skin Irritant and Tumor Promoting Diterpene Esters of the Tigliane Type from the Chinese Tallow Tree ( <i>Sapium sebiferum</i> ) . . . . .	199
Nomura, T., Fukai, T., Shimada, T., Ih-Sheng Chen: Components of Root Bark of <i>Morus australis</i> . I. Structure of a New 2-Arylbenzofuran Derivative, Mulberrofuran D. . . . .	90	Sepulveda-Boza, S., Friedrichs, E., Puff, H., Breitmaier, E.: Ein iso-Homoprotoberberin-Alkaloid aus den Wurzeln von <i>Berberis actinacantha</i> (An iso-Homoprotoberberin-Alkaloid from the Roots of <i>Berberis actinacantha</i> ) . . . . .	32
Obata-Sasamoto, H., Komamine, A.: Effect of Culture Conditions on DOPA Accumulation in a Callus Culture of <i>Stizolobium hassjoo</i> . . . . .	120	Shoyama, Y., Hatano, K., Nishioka, I.: Clonal Multiplication of <i>Pinellia ternata</i> by Tissue Culture . . . . .	14
Ohiri, F. C., Verpoorte, R., Baerheim Svendsen, A.: <sup>1</sup> H NMR Chemical Shift Values for Aromatic Protons in 2,3,9,10- and 2,3,10,11-tetrasubstituted Tetrahydroprotoberberine Alkaloids . . . . .	162	Stoianova-Ivanova, B., Budzikiewicz, H., Koumanova, B., Tsoutsoulova, A., Mladenova, K., Brauner, A.: Essential Oil of <i>Chrysanthemum indicum</i> . . . . .	236
Ohiri, F. C., Verpoorte, R., Baerheim Svendsen, A.: Tertiary Phenolic Alkaloids from <i>Chasmanthera dependens</i> . . . . .	17	Teh-Chang Chiang, Hson-Mou Chang, Mak, Th. C. W.: New Oleanene-type Triterpenes from <i>Abrus precatorius</i> and X-ray Crystal Structure of Abrusgenic Acid-Methanol 1:1 Solvate . . . . .	165
Ojewole, J. A. O., Adesina, S. K.: Mechanism of the Hypotensive Effect of Scopoletin Isolated from the Fruit of <i>Tetrapleura tetraptera</i> . . . . .	46	van der Sluis, W. G., van der Nat, J. M., Spek, A. L., Ike-shiro, Y., Labadie, R. P.: Gentiogenal, a Conversion Product of Gentiopicrocin (Gentiopicroside) . . . . .	211
Ojewole, J. A. O., Adesina, S. K.: Cardiovascular and Neuromuscular Actions of Scopoletin from Fruit of <i>Tetrapleura tetraptera</i> . . . . .	99	Wagner, H., Schwarting, G., Varljen, J., Bauer, R., Hamdard, M. E., El-Faer, M. Z., Beal, J.: Die chemische Zusammensetzung der Convolvulaceen Harze IV. Die Glykosidsäuren von <i>Ipomoea quamoclit</i> , <i>I. lacunosa</i> , <i>I. pandurata</i> und <i>Convolvulus al-sirensis</i> (Chemical Constituents of the Convolvulaceae-Resins IV. The Glycosidic Acids of <i>Ipomoea quamoclit</i> , <i>I. lacunosa</i> , <i>I. pandurata</i> and <i>Convolvulus al-sirensis</i> ) . . . . .	154
Okogun, J. I., Adeboye, J. O., Okorie, D. A.: Novel Structures of two Chromone Alkaloids from Root-Bark of <i>Schumanniohyton magnificum</i> . . . . .	95	Watanabe, K., Watanabe, H., Goto, Y., Yamaguchi, M., Yamamoto, N., Hagino, K.: Pharmacological Properties of Magnolol and Hönokiol Extracted from <i>Magnolia officinalis</i> : Central Depressant Effects . . . . .	103
Palacios, P., Gutkind, G., Rondina, R. V. D., de Torres, R., Coussio, J. D.: Genus <i>Baccharis</i> . II. Antimicrobial Activity of <i>B. crispa</i> and <i>B. notoserigila</i> . . . . .	128	Willuhn, G., Röttger, P.-M., Matthiessen, U.: Helenalin- und 11,13-Dihydrohelenalinester aus Blüten von <i>Arnica montana</i> (Helenalin- and 11,13-Dihydrohelenalinester from Flowers of <i>Arnica montana</i> ) . . . . .	226
Pandey, V. B., Singh, J. P., Mattocks, A. R., Bailey, E.: A Note on "Isolation and Pharmacological Action of Heliotrine, the Major Alkaloid of <i>Heliotropium indicum</i> Seeds" . . . . .	254	Witte, L., Berlin, J., Wray, V., Schubert, W., Kohl, W., Höfle, G., Hammer, J.: Mono- and Diterpenes from Cell Cultures of <i>Thuja occidentalis</i> . . . . .	216
Pathak, V. P., Saini, T. R., Khanna, R. N.: A New Furano-flavone from Seeds of <i>Pongamia glabra</i> . . . . .	61	Yang Minghe, Chen Yanyong: Steroidal Sapogenins in <i>Dioscorea colletii</i> . . . . .	38
Perera, P., Sandberg, F., van Beek, T. A., Verpoorte, R.: Tertiary Indole Alkaloids of <i>Tabernaemontana dichotoma</i> Seeds . . . . .	28	Yu De-Quan, Das, B. C.: Structure of Hydroxymuscovopyridine A and Hydroxymuscovopyridine B, Two New Constituents of Musk . . . . .	183
Perera, P., van Beek, T. A., Verpoorte, R.: Dichomine, a Novel Type of Iboga Alkaloid . . . . .	232	Yu De-quan, Y., Das, B. C.: Alkaloids of <i>Aconitum barbatum</i> . . . . .	85
Ravid, U., Putievsky, E.: Constituents of Essential Oils from <i>Majorana syriaca</i> , <i>Coridothymus capitatus</i> and <i>Satureja thymbra</i> . . . . .	248		
Röder, E., Wiedenfeld, H., Hönig, A.: Pyrrolizidinalkaloide aus <i>Senecio aureus</i> . . . . .	57		

**Subject Index 49, 1983****A**

Abietane 220  
Abrusgenic acid 165  
Abruslactone 165  
Acevaltrate 64, 191  
Adaptogenes 240  
Alkaloids 17, 20, 25, 28, 32, 43, 55, 57, 62, 79, 85, 95, 109, 124, 126, 131, 158, 162, 188, 196, 232, 244, 252, 254  
Amyrin 177  
Antineoplastic agents 115  
Antiviral agents 109  
Apigenin 128  
Aporphine alkaloids 20  
Aporphine derivatives 43  
Apparicine 232  
Arabinose 149  
Arylbenzofuran derivatives 90  
Avenasterol 177

**B**

Benzylisoquinoline alkaloids 131  
Berberine alkaloids 32  
Biflavones 204  
Bilobetin 204  
Biosynthesis of alkaloids 131, 196  
Biosynthesis of ajmalicine 79  
Biosynthesis of loganin 79  
Biosynthesis of sterols 176  
Biosynthesis of triterpenoids 176  
Biotransformation 9  
Bisabolol 67  
Bisbenzylisoquinoline alkaloids 20, 25, 55  
Bisnorargemonine 14  
Borneol 236  
Bornyl acetate 236

**C**

Cadambine 188  
Caffeic acid 186  
Camphor 216  
Cannabinoids 250  
Cardenolides 74  
Carvacrol 216  
Catechin 181  
Catechinxylopyranoside 181  
Cell cultures 9, 14 79, 115, 120, 131, 176, 191, 196, 216  
Chamazulene 67  
Cholestanol 177  
Chromone alkaloids 95  
Chrysanthenone 236  
Chrysosplenetin 63  
Cinchonamine 244  
Cinnamic acid 186  
Clonal multiplication 14

Cocarcinogens 3  
Convolvulaceae resins 154  
Coreximine 14  
Coronaridine 28  
Coumaric acid 186  
Coumarins 46, 99  
Cucurbitacin 176  
Curcumin 185  
Curcuminoids 185  
Cyanogenic glycosides 143  
Cycloartenol 177  
Cymenol 216  
Cynarin 224  
Cynaropicrin 246  
Cytotoxic action 158

**D**

Daphnane derivatives 3  
Dauricine 20  
Daurisoline 20  
Dehydrohelenalinester 226  
Deoxyphorbol 199  
Dichomine 232  
Didrovaltrate 64, 138, 191  
Diginatigenin derivatives 74  
Digoxigenin derivatives 74  
Dihydrocadambine 188  
Dihydroquinamine 244  
Diodantunezone 63  
Diosgenin 38  
Diterpene esters 3, 199  
Diterpenes 216  
Diterpenoid alkaloids 85  
Dopa 120

**E**

Enzymes 196  
Epilumicin 143  
Epoxyphorbol 199  
Erythrocentaurin 212  
Essential oils 9, 67, 216, 236, 248

**F**

Fenchone 220  
Ferruginol 220  
Ferulic acid 186  
Flavonoids 61, 63, 90, 128, 204  
Fluorocarpamine 124  
Fridelin 60  
Furoflavones 61

**G**

Galactose 149  
Genkwanin 128

Gentiogenal 211  
Gentiopicral 208  
Gentiopicrin 211  
Gentiopicroside 208, 211  
Geranial 79  
Geraniol 79  
Ginkgetin 204  
Glycosidic acids 154  
Glycyrrhetic acid 224  
Glycyrrhizin 224  
Govanine 17  
Guajazulene 67  
Guattegaumerine 25

**H**

Helenalin 226  
Helenalinester 226  
Helenanolides 226  
Heliotrine 254  
Hepatoma cells 138  
Hibiscone 127  
Hibiscoquinone 127  
Hinikione 220  
Hinokiol 220  
Hippadine 252  
Homovaltrate 64, 191  
Honokiol 103  
Huangshanine 55  
Hydroxymuscopryridine 183  
Hydroxymusizin glycoside 36

**I**

Iboga alkaloids 232  
Ibogamine 28  
Iboxygaine 232  
Immobilized cells 9  
Indicine 254  
Indole alkaloid glucoside 188  
Indole alkaloids 28, 62, 79, 124, 158, 188, 232  
Iridoids 208, 211  
Isochinoline alkaloids 196  
Isodihydrocadambine 188  
Isoginkgetin 204  
Isoquinoline alkaloids 32  
Isothujone 220  
Isovaltrate 64, 191

**K**

Kuwanon 90

**L**

Lasiocarpine 254  
Laudanosoline 196

Levomenol 67  
Liliolide 240  
Lindolhamine 20  
Linoleic acid 220  
Liriodenine 20  
Lumicin 143  
Lycorine 109

**M**

Magnolol 103  
Mandelonitril glycosides 143  
Matricine 67  
Melionine 158  
Mentenediol 220  
Menthone 9  
Methionine 224  
Methylabrusgenat 165  
Methylnorlaudanoline 196  
Methyltransferase 131, 196  
Moenjodaramine 126  
Monoterpenes 216  
Morphinane derivatives 43  
Morusin 90  
Mucilage 149  
Myrtanol 216

**N**

Naphthalene glycosides 36  
Naphthaquinones 63  
Neolignane derivatives 103  
Neomenthol 9  
Neral 79  
Nerol 79  
Norlaudanoline 131  
Normacusine 62  
Nororientaline 196  
Norreticuline 131

**O**

Opium alkaloids 131  
Orientaline 196

**P**

Pallidine 14  
Palmitic acid 220  
Papaver alkaloids 43  
Pedicellatin 240

Pedicellin 240  
Penduletin 63  
Perakine 62  
Perivine 232  
Phorbol 199  
Phytosterols 60, 176  
Piceid 51  
Picroside 224  
Pimelea factors 3  
Pleiocarpamine 124  
Podophyllotoxin 224  
Polysaccharides 149  
Proanthocyanidins 170  
Protoberberin derivatives 32  
Protoberberine alkaloids 20  
Protocatechuic acid 186  
Pseudoguaianolides 226, 246  
Puberaconitidine 85  
Puberaconitine 85  
Puberanidine 85  
Puberanine 85  
Pulegone 9  
Pyrrolizidine alkaloids 57

**R**

Resins 154  
Resveratrol 51  
Reticuline 20, 131  
Rhamnose 149  
Rhoeadine derivatives 43

**S**

Sabinene 220  
Sapogenins 38  
Saponins 38, 60  
Sarpagine 62  
Schumannificine 95  
Sciadopitysin 204  
Scopoletin 46, 99  
Secoiridoids 208, 211  
Sesquiterpene lactones 226, 246  
Sesquiterpenes 127  
Silybin 224  
Simplexin 3  
Sinapic acid 186  
Sitosterol 220  
Skin irritants 199  
Spinasterol 177  
Stemmadenine 28  
Stepholidine 20  
Steroidal alkaloids 126

Steroidal sapogenins 38  
Stigmastenol 177  
Stilbene 51  
Sugiol 220  
Swertiamarin 240

**T**

Tabersonine 28  
Tacaman alkaloids 232  
Tannins 181  
Taraxerol 60  
Terpineol 216  
Tertiary phenolic alkaloids 14  
Tetrahydroalstonine 62  
Tetrahydroisochinoline alkaloids 196  
Tetrahydroprotoberberine alkaloids 162  
Thalifaberine 55  
Thalifabine 55  
Thujaplicin derivatives 216  
Thujone 220  
Tigliane derivatives 3  
Tinnevellin glycoside 36  
Triptolide 109  
Triterpenoids 60, 165  
Tropolone 220  
Tumor promoters 199

**U**

Ursolic acid 240  
Ushinsunine 20

**V**

Valepotriates 64, 138, 191  
Valtrate 64, 138, 191  
Vellosimine 62  
Vindoline derivatives 124  
Vinorine 62  
Voacangine 28  
Voacristine 232  
Voaphylline 28, 232  
Vobasine 232

**Y**

Yamogenin 38

**Z**

Zierinxylolide 143

## Index of Names of Organisms 49, 1983

Please note that the following plant families are listed as indicated below:

Alsinaceae sub *Caryophyllaceae*

Apiaceae sub *Umbelliferae*

Arecaceae sub *Palmae*

Asteraceae sub *Compositae*

Brassicaceae sub *Cruciferae*

Clusiaceae sub *Guttiferae*

Fabaceae sub *Leguminosae* (including Mimosoideae = *Mimosaceae*, Caesalpinoideae = *Caesalpiniaceae* and Papilionoideae = *Papilionaceae* = Fabaceae sensu stricto)

Hypericaceae sub *Guttiferae*

Lamiaceae sub *Labiatae*

Oenotheraceae sub *Onagraceae*

Poaceae sub *Gramineae*

### A

*Abrus cantoniensis* 165  
*Abrus precatorius* 165  
*Aconitum barbatum* 85  
*Adlumia fungosa* 133  
*Albertisia papuana* 22  
*Alstonia yunnanensis* 62  
*Althaea officinalis* 152  
*Amaryllidaceae* 109, 252  
*Annonaceae* 20, 25  
*Anthemis altissima* 143  
*Anthemis cairica* 143  
*Anthocephalus cadamba* 189  
*Apocynaceae* 28, 62, 79, 124, 232  
*Araceae* 14  
*Argemone intermedia* 133  
*Argemone platyceras* 131, 196  
*Arnica montana* 226  
*Artemisia caruthii* 67

### B

*Baccharis crispa* 128  
*Baccharis megapotamica* 116  
*Baccharis notoserigila* 128  
*Baliospermum montanum* 200  
*Berberidaceae* 32, 133  
*Berberis actinacantha* 32  
*Berberis henryana* 133  
*Berberis stolonifera* 133  
*Berberis wilsonae* 133  
*Betula species* 181  
*Betulaceae* 181  
*Blackstonia perfoliata* 211  
*Bombax malabaricum* 127  
*Boraginaceae* 254  
*Brucea antidysenterica* 116  
*Bryonia dioica* 176  
*Buxaceae* 126  
*Buxus papillosa* 126

### C

*Cacalia floridana* 57  
*Caesalpinia gilliesii* 116  
*Caesalpiniaceae* 36  
*Campanulaceae* 60  
*Cannabis sativa* 250  
*Cassia angustifolia* 36  
*Cassia senna* 36  
*Catharanthus roseus* 79, 124  
*Celastraceae* 115  
*Cephalotaxaceae* 115  
*Cephalotaxus harringtonia* 115  
*Chamomilla recutita* 67  
*Chasmanthera dependens* 17, 162  
*Chelidonium majus* 133  
*Chlorella ellipsoidea* 178  
*Chrysanthemum indicum* 236  
*Cissampelos mucronata* 133  
*Clivia miniata* 109  
*Codonopsis pilosula* 60  
*Colchicum speciosum* 116  
*Compositae* 57, 67, 128, 143, 226, 236, 246, 255  
*Convolvulaceae* 154  
*Convolvulus al-sirensis* 154  
*Convolvulus microphyllus* 154  
*Coridothymus capitatus* 248  
*Corydalis pallida* 133  
*Corydalis sempervirens* 133  
*Crassocephalum multicorymbosum* 255  
*Crepis capillaris* 246  
*Crepis virens* 246  
*Crinum asiaticum* 252  
*Crinum augustum* 252  
*Crinum latifolium* 252  
*Crinum pratense* 252  
*Cucumis sativus* 176  
*Cucurbita maxima* 176  
*Cucurbita pepo* 177  
*Cucurbitaceae* 176  
*Cupressaceae* 170, 216  
*Curcuma longa* 185

### D

*Dictiostelium discoideum* 177  
*Digitalis lanata* 74  
*Digitalis schischkinii* 74  
*Dioscorea collettii* 38  
*Dioscoreaceae* 38

### E

*Erythrina lithosperma* 22  
*Eschscholtzia tenuifolia* 133  
*Euphorbiaceae* 199

### F

*Fagara zanthoxyloides* 116  
*Fumaria officinalis* 133

### G

*Gentiana pedicellata* 240  
*Gentianaceae* 208, 211, 240  
*Ginkgo biloba* 204  
*Ginkgoaceae* 204  
*Glaucium flavum* 133  
*Guatteria gaumeri* 25

### H

*Heliotropium indicum* 116, 254  
*Heliotropium supinum* 254  
*Hibiscus species* 127  
*Holacantha emoryi* 116  
*Hura crepitans* 3

### I

*Ipomoea lacunosa* 154  
*Ipomoea operculata* 154  
*Ipomoea pandurata* 154  
*Ipomoea quamoclit* 154  
*Ipomoea turpethum* 154  
*Isertia hypoleuca* 244

### J

*Juniperus communis* 170

### K

*Kydia calycina* 127

### L

*Labiatae* 9, 248  
*Lantana achyranthifolia* 63  
*Leguminosae* 36, 46, 61, 99, 120, 165  
*Lindera oldhamii* 22, 26  
*Loganiaceae* 158

### M

*Magnolia obovata* 103  
*Magnolia officinalis* 103  
*Magnoliaceae* 103  
*Majorana syriaca* 248  
*Malvaceae* 127

Maytenus buchananii 116  
Menispermaceae 17, 133, 162  
Menispermum dauricum 22  
Mentha species 9  
Mimosaceae 46, 99  
Moraceae 90  
Morus alba 90  
Morus australis 90  
Moschus moschiferus 183  
Mycobacterium species 10

**N**

Nicotiana tabacum 250

**O**

Ochrosia moorei 116  
Origanum compactum 249  
Origanum floribundum 249  
Origanum hirtum 249  
Origanum majorana 249  
Origanum maru 248  
Origanum smyrnaeum 249  
Origanum syriacum 248  
Orobanchaceae 250

**P**

Panax ginseng 60  
Panax pseudo-ginseng 60  
Papaver armeniacum 43  
Papaver cylindricum 43  
Papaver fugax 43  
Papaver orientale 196  
Papaver somniferum 131, 196  
Papaver tauricola 43  
Papaver triniifolium 43  
Papaveraceae 43, 131, 196  
Papilionaceae 120

Penicillium expansum 211  
Phelipaea ramosa 250  
Pimelea linifolia 7  
Pimelea prostrata 3  
Pimelea simplex 3  
Pinellia ternata 14  
Podophyllum hexandrum 223  
Polyalthia beccarii 20  
Polyalthia emarginata 20  
Polyalthia nitidissima 20  
Polyalthia oligosperma 20  
Polyalthia oliveri 20  
Polyalthia suaveolens 20  
Polygonaceae 51  
Polygonum cuspidatum 51  
Polygonum multiflorum 51  
Polypodium vulgare 181  
Pongamia glabra 61  
Putterlickia verrucosa 115

**R**

Ranunculaceae 55, 85, 133  
Rhus coriaria 248  
Rubiaceae 95, 188, 244

**S**

Sapium sebiferum 199  
Satureja capitata 248  
Satureja peltieri 249  
Satureja thymbra 248  
Schistosoma japonicum 199  
Schumanniophyton magnificum 95  
Schumanniophyton problematicum 97  
Scolytus multistriatus 181  
Scolytus ratzeburgi 181  
Scrophulariaceae 74  
Senecio aureus 57  
Stizolobium hassjoo 120  
Streptomyces clavuligerus 10

Strychnos melinoniana 158  
Strychnos usambarensis 158

**T**

Tabernaemontana dichotoma 28, 232  
Tabernaemontana eglandulosa 232  
Taxus brevifolia 116  
Tetrapleura tetraptera 46, 99  
Thalictrum dasycarpum 116  
Thalictrum faberi 55  
Thalictrum sparsiflorum 133  
Thalictrum tuberosum 133  
Thuja occidentalis 216  
Thymelaeaceae 3  
Thymus capitatus 248  
Tilia species 149  
Tiliaceae 149  
Tripterygium wilfordii 115

**U**

Ulmus americana 181  
Uncaria sinensis 188  
Uncaria species 188

**V**

Valeriana edulis ssp. procera 64  
Valeriana kilimandscharica 64  
Valeriana thalictroides 64  
Valeriana wallichii 138, 191  
Valerianaceae 64, 138, 191  
Verbenaceae 63

**Z**

Zingiberaceae 185

## Pharmacology Index 49, 1983

Biological Systems Organs / Diseases	Pharmacological Effects/ Effects on	Plant / Constituent	Page
Central nervous system	anticonvulsive	Magnolia officinalis, Magnoliaceae	103
	muscle relaxant	Magnolia officinalis	103
	sedative	Magnolia officinalis	103
Peripheral nervous system	muscle relaxant	Tetrapleura tetraptera, Mimosaceae	99
Autonomous nervous system	spasmolytic	Tabernaemontana dichotoma, Apocynaceae	28
Cardiovascular system	hypotensive	Tetrapleura tetraptera, Mimosaceae	46
	hypotensive	Tetrapleura tetraptera, Mimosaceae	99
Hormonal system	antifertility	Hippadine, Amaryllidaceae	252
Infectious diseases	antibacterial	Baccharis crispa, Asteraceae	128
	antiviral	Clivia miniata, Amaryllidaceae	109
Tumors	tumor promotion	Pimelea simplex, Thymelaeaceae	3
Inflammation	antiinflammatory	Chamomilla reculita, Asteraceae	67
	antiinflammatory	Crassocephalum multicorymbosum	255

# Partial Purification and Properties of S-Adenosylmethionine: (R), (S)-Norlaudanosoline-6-O-Methyltransferase from *Argemone platyceras* Cell Cultures

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Received: August 7, 1983; accepted: August 31, 1983

## Key Word Index:

*Argemone platyceras*; Papaveraceae; Cell Suspension Cultures; Alkaloid Biosynthesis; S-Adenosylmethionine: (R), (S)-Norlaudanosoline-6-O-Methyltransferase.

## Abstract

A new enzyme, S-adenosylmethionine: (R), (S)-norlaudanosoline-6-O-methyltransferase, was isolated from the soluble protein extract of *A. platyceras* cell cultures and purified approximately 80-fold. This enzyme catalyses the formation of 6-O-methylnorlaudanosoline, and, to a minor extent, 7-O-methylnorlaudanosoline from SAM and (S), as well as (R), norlaudanosoline. The apparent molecular weight of the enzyme is 47000 Dalton. The pH-optimum of the enzyme is 7.5, the temperature optimum, 35° C. Apparent  $K_M$  values for (S) and (R)-norlaudanosoline were 0.2 mM, and for SAM, 0.05 mM. The transferase shows high substrate specificity for tetrahydrobenzylisoquinoline alkaloids. Simple orthophenols, like phenylpropane derivatives, coumarins or flavonoids, are not accepted as substrates. The enzyme is widely distributed in benzylisoquinoline-containing plant cell cultures and is present in differentiated plants like *Papaver somniferum*.

## Introduction

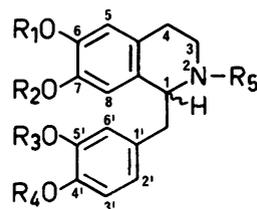
(S)-NLS is now firmly established as the first intermediate in benzylisoquinoline alkaloid biosynthesis [1]. A recently discovered enzyme [2] catalyses its

formation from dopamine and 3,4-dihydroxyphenylacetaldehyde.

Further down the pathway, reticuline is proven to be a branch point intermediate in the biosynthesis of a vast array of structure types of benzylisoquinoline alkaloids, as for instance: morphinanes, protoberberines, proaporphines, cularines, dibenzopyrrocolines etc. (e.g. 3]. This means that NLS must be transformed to reticuline by three methylation reactions, two O-methylations at positions 6 and 4' and one N-methylation (at atom 2). Since nor-reticuline has been amply demonstrated by feeding experiments to be a precursor of reticuline *in vivo* [4, 5], one has to assume that O-methylation of NLS precedes N-methylation for instance in opium alkaloids. O-Methylation of NLS is therefore an important reaction in the early steps of the biosynthesis of reticuline, the universal branch point intermediate. O-Methyltransferases have been reported to mediate the transfer of methyl groups from SAM to phenylpropanoid and flavonoid compounds mainly at the *meta* position of the aromatic system, though *para*-O-methylation is not uncommon [6 and literature cited therein]. Specific-O-methyltransferases derived from plants and acting on tetrahydrobenzylisoquinolines have so far not been reported. Yet incubation of poppy latex with (R,S)-norlaudanosoline and  $^{14}\text{C}$ -SAM has led to the formation of labelled opium alkaloids, thus demonstrating indirectly the presence of methyltransferase enzymes [7]. There are, however, several reports on NLS-O-methylation, by animal enzymes [e.g. 8].

In our attempt to elucidate the enzymatic steps involved in isoquinoline biosynthesis in plants, we have investigated the O-methylation of NLS. In this report, we present the partial purification and properties of a new enzyme named norlaudanosoline-6-O-methyltransferase from *A. platyceras* cell suspension cultures. This enzyme catalyses the predominant transfer of the S-methyl group of SAM to the phenolic OH-group at position 6 of NLS, and to a lesser extent, to the 7-O-position.

Abbreviations: NLS = Norlaudanosoline; SAM = S-Adenosyl-L-methionine; SAH = S-Adenosylhomocysteine, NLS-OMT = S-Adenosylmethionine: (R), (S)-Norlaudanosoline-6-O-Methyltransferase.



	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>
Norlaudanosoline	H	H	H	H	H
6-O-methylnorlaudanosoline	CH <sub>3</sub>	H	H	H	H
7-O-methylnorlaudanosoline	H	CH <sub>3</sub>	H	H	H
5-O-methylnorlaudanosoline	H	H	CH <sub>3</sub>	H	H
4'-O-methylnorlaudanosoline	H	H	H	CH <sub>3</sub>	H
Laudanosoline	H	H	H	H	CH <sub>3</sub>
Norreticuline	CH <sub>3</sub>	H	H	CH <sub>3</sub>	H
Nororientaline	CH <sub>3</sub>	H	CH <sub>3</sub>	H	H
Laudanidine	CH <sub>3</sub>	CH <sub>3</sub>	H	CH <sub>3</sub>	CH <sub>3</sub>

1H =  $\alpha$  (S); 1H =  $\beta$  (R)

## Materials and Methods

### Plant Material

*A. platyceras* cell culture was initiated and maintained since 1975 on Linsmeyer and Skoog (LS) medium [9]. Batch cultures in 1 litre Erlenmeyer flasks containing 250 ml medium were agitated on a gyratory shaker (100 rpm) in diffuse light (750 lux) at 24° C and were subcultured at weekly intervals using about 10% inoculum. The cells were harvested after 8 days, frozen in liquid nitrogen and stored at -20° C. Cell fresh weight was determined after filtering the cells through a fritted glass funnel using suction. Aliquots were used for dry-weight determination. All other cell cultures were from our culture collections and were grown under identical conditions as given above.

### Chemicals

The following compounds were obtained from the indicated sources: S-adenosyl-L-methionine hydrogen sulphate, Combithek (calibration proteins), S-adenosylhomocysteine, all from Boehringer, Mannheim; S-adenosyl-L-methyl-<sup>3</sup>H-methionine was prepared enzymatically from methionine-S-methyl-<sup>3</sup>H, (Radiochemical Centre Amersham) using standard methods. All benzylisoquinoline alkaloids were synthesized according to standard procedures. ACA 34 was purchased from LKB, DEAE-cellulose-microgranular form from Whatman, and hydroxyapatite from Bio-Rad. All other materials were of reagent grade. Liquid scintillation counting was performed in a toluene mixture (Rotiszint 22, Roth).

### O-Methyltransferase Assay and Product Identification

During enzyme purification, OMT activity was assayed against R/S-norlaudanosoline, except that with crude enzyme preparations, laudanosoline was substituted for its nor-derivative. The assay mixture consisted of KPO<sub>4</sub><sup>2-</sup>-buffer, pH 7.5 (130 mM), ascorbate (130 mM) R/S-norlaudanosoline (0.3 mM), <sup>3</sup>H-SAM (0.1 mM, 10000 cpm), and varying amounts of enzyme in a total volume of 150  $\mu$ l.

The mixture was incubated for 45 minutes at 35° C and the reaction was terminated by addition of 200  $\mu$ l Na<sub>2</sub>CO<sub>3</sub>-buffer (1 M, pH 9.5). The methylated products were extracted by adding 400  $\mu$ l isoamylalcohol and shaking for 45 min. The turbid mixture was cleared by centrifugation in an Eppendorf centrifuge for 5 min. 200  $\mu$ l of the organic phase were transferred to scintillation vials and counted for radioactivity. Recovery of the methylated products was 95% under these conditions. Blank mixtures containing either

no enzyme or no substrate (NLS) yielded a blank value of about 8% radioactivity, a value which was subtracted from all incubation mixtures.

For product identification, the products were separated by HPLC using a Nucleosil-SA-column (25 mm  $\times$  3.2 mm i.d.) and 0.5 ammonium phosphate : methanol (80:20) as a solvent system. Retention times of the potential products were: 5'-O-methyl-NLS, 6.45 min.; 7-O-methyl-NLS, 7.35 min.; 4'-O-methyl-NLS, 8.31 min.; 6-O-methyl-NLS, 9.18 min. Preparative isolations were done by using the above incubation mixture  $\times$  100. The reaction product was extracted by ethylacetate and subjected to TLC (Sigel; solvent system: Chloroform : methanol : acetic acid : water = 18:6:3:0.3). The zone containing radioactivity was scraped off, purified for a second time in the solvent system: Chloroform : n-propanol : methanol : water = 45:15:60:40 (CHCl<sub>3</sub>-phase), and its mass spectrum was measured in a Finnigan MAT 44S instrument.

### Enzyme purification

**Step 1:** 100 g frozen tissue was allowed to thaw in 0.1 M KPO<sub>4</sub><sup>2-</sup>-buffer, pH 7.5, containing 20 mM  $\beta$ -mercaptoethanol, stirred for 20 minutes, pressed through cheese cloth, and centrifuged at 48000 xg for 10 min. Ammoniumsulphate precipitation was done from 0-70% saturation, centrifuged again for 10 min. at 48000 xg. The pellet was taken up in 0.1 M KPO<sub>4</sub><sup>2-</sup>-buffer, pH 7.5, containing 20 mM  $\beta$ -mercaptoethanol.

**Step 2:** The crude extract from step 1 (15 ml) was put on an Ultrogel ACA 34 column (l = 90 cm,  $\phi$  = 2.5 cm), equilibrated with 10 mM KPO<sub>4</sub><sup>2-</sup>-buffer, pH 7.5, 20 mM  $\beta$ -mercaptoethanol. Fractions of 4 ml were collected at a flow rate of 8 ml/h. The fractions (45-61) containing the enzyme activity were pooled and subjected to the next step.

**Step 3:** The fractions containing the enzyme (59 ml), were subjected to ionexchange chromatography on DEAE-cellulose (l = 10 cm,  $\phi$  = 1.5 cm). The column was equilibrated with 10 mM KPO<sub>4</sub><sup>2-</sup>-buffer, pH 7.5, containing 20 mM  $\beta$ -mercaptoethanol, and a gradient was applied from 0-300 mM KCl (8 hrs). 4 ml Fractions were collected at a flow rate of 40 ml/h. The enzyme was found in fractions 56-64. The fractions containing the enzyme were pooled and applied to the next step.

**Step 4:** The protein solution was added to a hydroxyapatite column (l = 10 cm,  $\phi$  = 1 cm), equilibrated with 10 mM KPO<sub>4</sub><sup>2-</sup>-buffer, pH 7.5, 20 mM  $\beta$ -mercaptoethanol, and a gradient of 10-200 mM KPO<sub>4</sub><sup>2-</sup>, pH 7.5, was applied (8 hrs). 2 ml Fractions were collected at a flow rate of 30 ml/h. Enzyme activity was found in frac-

tions 43–54 with NLS as a substrate. Soluble protein was determined as described previously [10] or in more highly purified samples, with an optical method [11].

#### Molecular Weight Determination

The molecular weight determination of the purified O-methyltransferase was carried out by gel filtration on a calibrated G-100-superfine column. Although only the Stokes radii of the proteins can be determined by this method, it is often used for the determination of the molecular weight assuming globular shape for the proteins. The column, 168 ml ( $l = 95$  cm,  $\phi = 1.5$  cm), equilibrated with 10 mM  $KPO_4^{2-}$ -buffer, pH 7.5, 20 mM  $\beta$ -mercaptoethanol, was eluted at a flow rate of 15 ml/h in 100 fractions of 2 ml. The column was calibrated with the proteins of the Combithek. Ferritin (MW: 450000) was used for the determination of the void volume of the column. The standards were monitored by the absorbance at 280 nm. The results are given as Stokes radii.

## Results

The presence of O-methyltransferases in crude enzyme extracts of plant cell cultures of different taxonomic origin was investigated using (R, S)-laudanosoline and  $C^3H_3$ -SAM as substrates. Laudanosoline was chosen as an initial methyl group acceptor to avoid interference with N-methyltransferases potentially present in the assay. Using these substrates in the standard assay, O-methyltransferases were detected in all cell cultures containing isoquinoline alkaloids thus far tested (Table I). Cell cultures of four different plant families tested for the presence of O-methyltransferase enzymes showed positive results. By far the highest absolute amount of O-methyltransferase per unit culture fluid was found in *Argemone platyceras* (Papaveraceae), a plant species known to contain isoquinoline alkaloids of the pavine, protoberberine, and aporphine types [12]. It was therefore decided to use cells of this species to purify and characterize the O-methyltransferase. (R, S)-norlaudanosoline was used as a substrate in the purification of the enzyme since it was possible to demonstrate that using this plant tissue, there was no interference with N-methyltransferases under the conditions chosen for the assay.

All of the NLS-methylating enzyme activity was detected in the 100000 xg supernatant of the homogenate of the *Argemone* cells. It could not be found in

**Table I**

Survey of distribution of (R, S)-laudanosoline-methyltransferase activity in species of different isoquinoline alkaloid containing families (laudanosoline served as substrate)

Plant material	Family	Enzyme activity pkat/l medium	pkat/mg protein
Cell cultures:			
<i>Argemone platyceras</i>	Papaveraceae	6350	13.8
<i>Corydalis sempervirens</i>	"	4620	12.2
<i>Glaucium flavum</i>	"	2855	27.7
<i>Fumaria officinalis</i>	"	1950	11.1
<i>Corydalis pallida</i>	"	1480	8.2
<i>Argemone intermedia</i>	"	1360	33.7
<i>Adlumia fungosa</i>	"	1070	12.5
<i>Papaver somniferum</i>	"	1020	9.3
<i>Chelidonium majus</i>	"	915	6.8
<i>Eschscholtzia tenuifolia</i>	"	590	4.2
<i>Berberis henryana</i>	Berberidaceae	2050	38.7
<i>Berberis wilsonae</i>	"	1650	13.9
<i>Berberis stolonifera</i>	"	1260	18.9
<i>Thalictrum tuberosum</i>	Ranunculaceae	2060	26.6
<i>Thalictrum sparsiflorum</i>	"	570	2.4
<i>Cissampelos mucronata</i>	Menispermaceae	940	3.7
Differentiated plant:		pkat/gdwt	pkat/mg
<i>Papaver somniferum</i>		218	1.6

the culture filtrate. The enzyme was isolated and partly purified by ammonium sulphate precipitation, ACA 34, DEAE-cellulose, and hydroxyapatite chromatography as described under "Materials and Methods". This procedure yielded a purification of approximately 80-fold with a recovery of 13%. The data for a typical purification procedure are summarized in Table II. The protein solution at the stage of highest purification did not contain any other enzymes of the isoquinoline biosynthetic pathway thus far tested. A typical elution profile of a hydroxyapatite column is shown in Fig. 1.

#### Properties of the O-Methyltransferase

The 80-fold purified enzyme was used to determine the catalytic properties. The activity of the

**Table II**

Purification procedure for O-methyltransferase from *Argemone platyceras* (R, S)-norlaudanosoline as substrate)

Purification step	Total activity (pkat)	Total protein (mg)	Specific activity pkat/mg	Recovery (%)	Purification -fold
Crude extract	3626	259.6	14	100	1
Ammoniumsulfate-precipitation (0–70%)	3409	178.3	19	94	1.4
Gel filtration (ACA = 34)	2290	15.2	150	63.2	10
DEAE-cellulose chromatography	1124	2.5	459	31.0	32
Chromatography on hydroxyapatite	477	0.4	1173	13.2	83

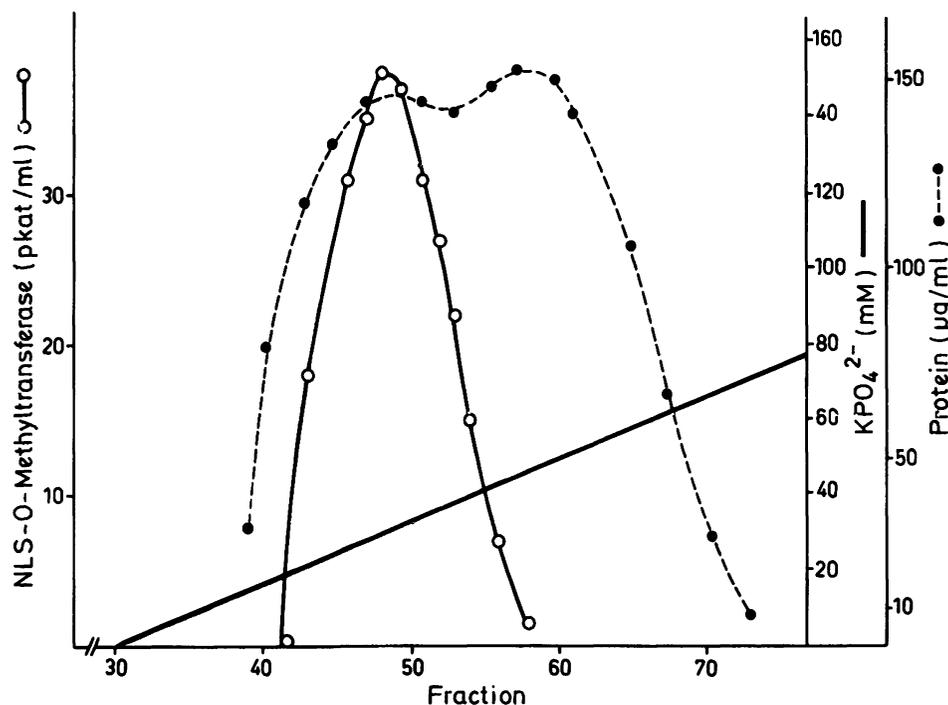


Fig. 1. Elution profile of NLS-OMT from a hydroxyapatite column.

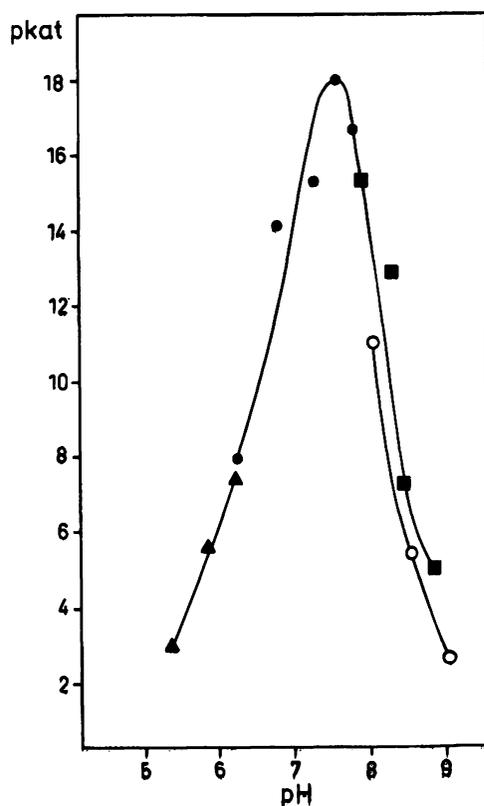


Fig. 2. pH profile of the catalytic activity of purified NLS-OMT from *A. platyceras* cell cultures. Buffers used: ▲ — ▲ Na-maleate/NaOH; ● — ● K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>; ■ — ■ Tris/HCl; ○ — ○ Na-borate.

enzyme was measured at a range of pH 5–9 with different buffers as shown in Fig. 2.

The O-methyltransferase from *Argemone* shows a clear pH-optimum at pH 7.5. The enzyme exhibits a temperature optimum at 35° C. The molecular weight of the O-methyltransferase determined by gel filtration on Sephadex G 100 was 47000 daltons. The enzyme was inhibited by preincubation for 15 min. at 35° C with the following metal ions added as sulphates to a final concentration of 5 mM, and the reaction started by the addition of the labelled substrate: Cu<sup>++</sup> (100% inhibition), Mn<sup>++</sup> (15%), Zn<sup>++</sup> (100%), Mg<sup>++</sup> (0%), Co<sup>++</sup> (81%), Ni<sup>++</sup> (100%), Sn<sup>++</sup> (55%), Hg<sup>++</sup> (100%), Fe<sup>++</sup> (90%). No inhibition was found after preincubation with 25 mM EDTA. Of the organic enzyme inhibitors tested only p-chloromercuribenzoate (25 mM, 70%) and iodobenzoic acid (10 mM, 73%) resulted in substantial inhibition of the enzyme. This indicates that there is an SH-group requirement for full enzyme activity. A competitive inhibition was observed using S-adenosyl-homocysteine in the presence of <sup>3</sup>H-SAM and NLS, as substrates. A K<sub>i</sub> of 10 µm was determined for SAH. The enzyme shows a half life of 8 hrs at 30° C. At 4° C after a 4 week storage period, 50% of the initial activity of the enzyme was lost. All enzyme activity was lost when the enzyme was frozen in 30% glycerol solution.

As shown in Table III, the purified enzyme is absolutely specific for the tetrahydrobenzylisoquinoline

Table III

Substrate specificity of partially purified NLS-OMT from *A. platyceras* cell cultures

Substrate	Enzyme pkat/mg enzyme	activity %*	Reaction product
(S)-Norlaudanoline	313	100	6-O-methylnorlaudanoline: 7-O-methylnorlaudanoline = 8:2
(R)-Norlaudanoline	288	92	same as above
(R, S) 4'-O-methylnorlaudanoline	107	34	Norprotosinomenine
(R, S) 5'-O-methylnorlaudanoline	255	81	Nororientaline
(R, S)-Laudanosoline	247	79	6-O-methylaudanosoline
(R, S)-Norlaudanoline-1-carboxylic acid	15	5	nd
(R, S)-Norreticuline	3	1	nd
(R, S)-Nororientaline	0	0	–
(R, S)-Laudanidine	0	0	–
(S)-Scoulerine	3	1	Tetrahydrocolumbamine
2,3-Dihydroxy-9,10-dimethoxy protoberberine	22	7	Jatrorrhizine
(R, S)-2,3-Dihydroxy-9,10-dimethoxy tetrahydroprotoberberine	15	5	Tetrahydrojatrorrhizine
Adrenaline	0	0	–
Aesculetine	0	0	–
Caffeic acid	0	0	–
Catechol	0	0	–
Dopamine	0	0	–
Quercetin	0	0	–

\* relative to (S)-NLS; nd = not determined

nucleus. None of the phenylpropanoids, phenolics, biogenic amines, coumarins or flavonoids were methylated by action of this enzyme. The best substrates were the ones with four free phenolic-hydroxy-functions, followed by mono-methyl-derivatives. Di-O-methylated alkaloids are very poor substrates.  $K_M$  values for this enzyme were determined using the following substrates:  $K_M$  (S)-norlaudanoline 0.2 mM; (R)-norlaudanoline 0.2 mM; (R,S)-4'-O-methylnorlaudanoline 1.1 mM; (R, S)-laudanoline 0.3 mM. SAM 0.05 mM (with (S)-NLS as substrate).

The 80-fold purified OMT is absolutely free of any N-methylating activity as demonstrated by its lack of activity on norreticuline, nororientaline or tetrahydropapaverine.

The best enzyme substrate of the compounds tested so far proved to be (S)-norlaudanoline. The product of the reaction catalysed by the transferase using (S)-NLS and  $^{14}\text{C}$ -SAM as substrates was subsequently investigated. HPLC-chromatography under the conditions given, showed that only two radioactive products were formed, one compound with a retention time of 7.2 min. (20 yield) and the major compound with a retention time of 9.2 min. (80 % yield). The retention times and co-chromatography with all four possible authentic monomethylated norlaudanolines indicated the minor compound to be 7-O-methyl-norlaudanoline and the major compound to be 6-O-methyl-norlaudanoline. Preparative isolation of the major compound from a 15 ml incuba-

tion mixture containing (R, S)-laudanoline as substrate, mass spectroscopy of the purified major unknown product showed a clear fragment (3,4-dihydro-6-methoxy-7-hydroxy-N-methyl-isochinolinium cation) with  $m/z$  192 (100 %) containing one N and one O-methyl-group which further fragmented to  $m/z$  177 (192- $\text{CH}_3$ , 30 %), and  $m/z$  162 (177- $\text{CH}_3$ , 4 %). The spectrum clearly indicated methylation at the A-ring and was identical in every respect with the mass spectrum of authentic 6-O-methylaudanosoline. Thus the major product of the NLS-OMT reaction was proven to be 6-O-methylnorlaudanoline.

The time course of the enzyme formation in suspension cultures of *Argemone* cells is shown in Fig. 3. The enzyme is present in the inoculum only in low amounts. The activity peaks at day 8 of cultivation exactly at the point when the culture is leaving the logarithmic growth phase. A 30-fold increase in activity can be seen as compared with only 10-fold increase in dry cell matter. During stationary phase there is a drastic decrease of total activity of the enzyme.

## Discussion

Isoquinoline alkaloids comprise the largest group of alkaloids in plant kingdom. Relatively little is known about their biosynthesis at the cell-free level. In an attempt to elucidate the enzymatic steps involved in benzyloisoquinoline synthesis, it is our primary

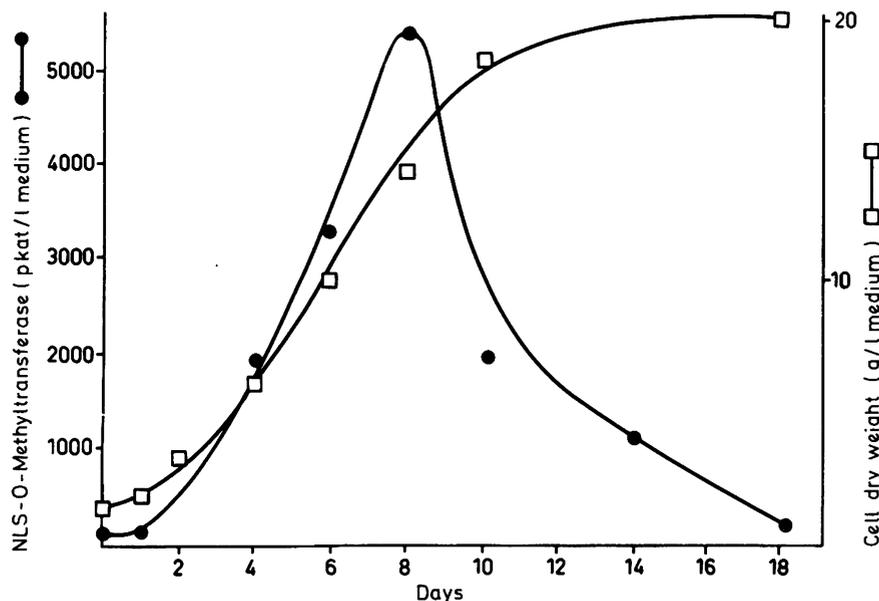


Fig. 3. Time course of NLS-O-methyltransferase activity and cell dry weight in a suspension culture of *A. platyceras*.

aim to isolate and characterize those enzymes which are involved in reticuline biosynthesis, the central and branch-point intermediate in benzyloquinoline metabolism in plants [3]. In previous studies, we were able to demonstrate that the initial reaction in formation of the benzyloquinoline skeleton is the stereospecific condensation of dopamine and 3,4-dihydroxyphenylacetaldehyde to yield (S)-norlaudanosoline [1, 2]. The aldehyde, rather than the 3,4-dihydroxyphenylpyruvate, is the substrate for this condensation reaction. The previously postulated intermediate norlaudanosoline-1-carboxylic acid [13–15] is most probably an artefact. Between the now recognized first metabolite in the pathway norlaudanosoline and reticuline, three methylation steps are involved, two O-methylations at carbon atom 6 and 4' and one N-methylation at atom 2. Since nor-reticuline has been amply demonstrated by *in vivo* experiments to be the immediate precursor of reticuline [e.g. 4, 5], the question arises, which of the phenolic groups of the norlaudanosoline molecule is methylated first on its way to nor-reticuline, the one in 6 or the one in 4' position. A survey of plant cell cultures using (R,S)-laudanosoline and  $^3\text{H}$ -SAM as substrates (in order to prevent N-methylation) demonstrated that all isoquinoline containing species contained good methylating activity. No methyltransferase activity was observed using (R,S)-NLS and  $^3\text{H}$ -SAM as substrate with for instance *Catharanthus roseus* (Apocynaceae), a species which is known *not* to contain any benzyloquinoline alkaloids. The highest amount of methyltransferase per volume of medium was observed in *A. platyceras* cell cultures, and it was decided to use this species for the isolation and characterization of the NLS-O-methyltransferase enzyme. The test used for the analysis of the methylated NLS formed involved differential extraction of the

labelled product at pH 9.5 with isoamylalcohol leaving residual  $^3\text{H}$ -SAM in the aqueous solution.

By using this assay the methylating enzyme could be purified about 80-fold and the major (80%) product of reaction could unequivocally be identified as 6-O-methylnorlaudanosoline by HPLC and mass spectroscopy and comparison with the other four authentic mono-methyl-NLS species. The minor reaction product (20%) was identified as 7-O-methyl-NLS. It is noteworthy that using the plant enzyme there was not much difference in the methylation rate using the stereoisomers (S)- or (R)-NLS. Furthermore there was no difference in methylation pattern observed using the pure optical isomers as substrates. In both cases 80% of the product formed was 6-O-methyl-NLS, formed regardless of whether (R)- or (S)-NLS was used as substrate. This demonstration is

Table IV

Comparison of Norlaudanosoline-O-methyltransferases of plant and animal origin

Characteristics	Enzyme source		
	<i>A. platyceras</i>	Rat [17]	Mouse*
(S)-NLS: $K_M$	0.2 mM	–	0.8 mM
(R)-NLS: $K_M$	0.2 mM	–	2.2 mM
(R, S)-NLS: $K_M$	–	1.3 mM	–
SAM: $K_M$	0.05 mM	6.2 mM	1.0 mM
pH-optimum	7.5	7.7–8.0	7.5
Position of methylation	6 and 7	6 and 7	6 and 7
Ratio for (S)-NLS	80:20	79:14	53:47
Ratio for (R)-NLS	80:20	26:68	47:53
Caffeic acid methylated	No	N.d.	Yes

\* This investigation was carried out in our laboratory using a mouse liver preparation according to [17]

