**Title:** Structure and biosynthesis of isatropolones, bioactive amine scavenging fluorescent natural products from Streptomyces Gö66

**Authors:** Helge B. Bode



**To be cited as:** 10.1002/ange.201701223

**Link to VoR:** https://doi.org/10.1002/ange.201701223

# Structure and biosynthesis of isatropolones, bioactive amine scavenging fluorescent natural products from *Streptomyces* Gö66

Xiaofeng Cai, Yi-Ming Shi, Nicole Pöhlmann, Ole Revermann, Isabel Bahner, Sacha J. Pidot, Frank Wesche, Helmut Lackner, Claudia Büchel, Marcel Kaiser, Christian Richter, Harald Schwalbe, Timothy P. Stinear, Axel Zeeck, Helge B. Bode*

Dr. X. Cai,[+] Dr. Y.-M. Shi,[+] M. Sc. F. Wesche, Prof. Dr. H. B. Bode, Merck Stiftungsprofessur für Molekulare Biotechnologie, Fachbereich Biowissenschaften, Goethe Universität Frankfurt, Max-von-Laue-Str. 9, 60438 Frankfurt am Main (Germany)

E-mail: h.bode@bio.uni-frankfurt.de

Homepage: http ://www.bio.uni-frankfurt.de/48050101/ak-bode

Dr. N. Pöhlmann, Dr. O. Revermann, Dr. I. Bahner, Prof. Dr. H. Lackner, Prof. Dr. A. Zeeck, Institut für Organische und Biomolekulare Chemie, Georg-August Universität Göttingen, Tammannstr. 2, 37077 Göttingen (Germany)

Dr. S.J. Pidot, Prof. Dr. T. P. Stinear, Department of Microbiology and Immunology,
The Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Melbourne (Australia)

Prof. Dr. Claudia Büchel, Institute of Molecular Biosciences, Goethe University Frankfurt, Max von Laue Str. 9, 60438 Frankfurt am Main (Germany)

Dr. M. Kaiser, Swiss Tropical and Public Health Institute, Parasite Chemotherapy, Socinstrasse 57, P.O. Box, 4002 Basel and University of Basel, Petersplatz 1, 4003 Basel (Switzerland)

Dr. C. Richter, Prof. Dr. H. Schwalbe, Institut für Organische und Chemische Biologie, Zentrum für Biomolekulare Magnetische Resonanz, Goethe Universität Frankfurt, Max-von-Laue-Str. 7, 60438 Frankfurt am Main (Germany)

Prof. Dr. H. B. Bode, Buchmann Institute for Molecular Life Sciences (BMLS), Goethe Universität Frankfurt, 60438 Frankfurt am Main (Germany)

[+] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under http://XXXX.

**Abstract**

The natural products isatropolone A-C (**1**-**3**) were reisolated from *Streptomyces* Gö66 with **1** and **3** showing potent activity against *Leishmania donovani*. They contain a rare tropolone ring derived from a type II polyketide biosynthesis pathway. Their biosynthesis was elucidated by labelling experiments, analysis of the biosynthesis gene cluster, its partial heterologous expression and structure elucidation of intermediates. Due to their 1,5-dione moiety they can react with ammonia, amines, lysine and lysine-containing peptides and proteins resulting in covalent binding and subsequent pyridine ring formation. Their fluorescence properties change upon amine binding allowing the simple visualisation of the reacted amines including the visualization of proteins.

## Introduction

Electrophilic natural products like Michael acceptors, ring-strained scaffolds like epoxides, lactones or lactams or other moieties often show a biological activity that is the result of their intrinsic reactivity with cellular nucleophiles like cysteine, serine, threonine or lysine residues.[1,2] Despite the success of such compounds since the early days of natural product research with for example β-lactams as one of the most widely used class of antibiotics, such compounds were almost neglected during the last years due to their often assumed high reactivity resulting in low selectivity. Luckily, this view is currently changing due to several compounds that show selectivity despite their electrophilic moiety[3] and because several synthetic electrophiles have been successfully brought to the clinic or are currently in clinical trials.[4-6] Additionally, their reactivity often allows the identification of their molecular target using modern chemical biology and proteomics approaches much easier than for non-covalent binding natural products.[7] This has not only clinical relevance but also contributes to our understanding of living systems in general.

There are also natural products that are so reactive that they react already in the producing organisms or the growth medium. Examples are the fungal azaphilones [8] that can react with different amines, amino acids or even peptides[9] resulting in the production of pigments or even potent blockers of protein-protein interaction. Similar compounds from bacteria are the rubrulones,[10,11] tropolone and pyridine ring containing natural products from *Streptomyces*. Very recently their biosynthesis has been investigated [11] and it has been shown that while the tropolone ring results from rearrangement of a type II polyketide synthase (PKS) pathway, the pyridine ring is formed non-enzymatically between an amine and the 1,5-dione moiety present in the rubrulone precursor.[12] However, the

amine free precursor has only been identified after manipulation of the biosynthesis gene cluster (BGC) resulting in the production of the putative aglycon.

Here we describe the identification and biosynthesis of isatropolones A-C (**1**-**3**),[13] natural products from *Streptomyces* Gö66 that besides having an unusual tropolone ring structure still show a 1,5-diketone structure that can efficiently form a pyridine ring with different amines including lysine residues from proteins. While binding to the amine moiety the isatropolone fluorophore changes allowing the simple visualization of the newly formed isarubrolone derivatives. Interestingly, isatropolones are potent and specific inhibitors of *Leishmania donovani* while the respective rubrulones are much less active, pointing to the covalent amide binding also as their mode of action.

Chemical screening of *Streptomyces* Gö66 revealed the presence of three yellowish red pigments **1**-**3**, which were produced in yields of 12, 6 and 3 mg/L in shaking flasks and in yields up to 50 mg/L in an airlift fermenter (Fig. S1).[13] Compounds **1** and **2** showed the same sum formula of $C_{24}H_{24}O_9$ as determined by HR-ESI/MS, **3** showed one oxygen atom more (Table S1). Details about the isolation and structure elucidation of **1**-**3** are only mentioned briefly here since they have been described in a PhD thesis[13]: Analysis of the NMR data ($^{1}H$, $^{13}C$, COSY, HSQC, HMBC, ROESY; Figs. S2and S3) of the main product **1** allowed the determination of its structure. While a methyl group, a butyryl moiety and 6-deoxy-D-allosylpyranosid as sugar moiety connected to a dihydrofuran ring could be readily identified and assigned (Fig. S2), the assignment of the remaining molecule was difficult due to the presence of eight quarternary $sp^2$ carbons among the remaining 10 carbons (Table S2). Compound **2** differed from **1** only in the configuration of C-3' in the sugar moiety and **3** carried the same sugar as **1** but a hydroxylated butyryl moiety (Fig. S3, Tables S3 and S4).

However, from subsequent HMBC experiments an unusual tropolone structure similar to that described in rubrolone A (**4**) and B (**5**) was suggested [11] (Fig. S2) for **1**-**3** although **4** and **5** showed an extra pyridine ring and a different sugar moiety. Since the rubrolones have been shown to be derived from a isatropolone-like precursor identified after deletion of the predicted glycosyltransferase,[12] **1**-**3** were incubated with ammonia leading to a clean conversion into the corresponding pyridine derivatives named isarubrolone A-C (**6**-**8**). The detailed NMR analysis of **6**-**8** (Tables S5-S7), analysis of acetate labelled and ammonia transformed **1** (Table S5, Fig. 1b) using also an INADEQUATE experiment confirmed all structures.

Bioactivity testing of **1**-**3** and **6**-**9** did not reveal any antimicrobial activity and only very weak cytotoxic activity was observed (Table 1) similar to the low cytotoxicand antioxidative activity described for **4**.[11] However, when these compounds were tested against different protozoa (*Trypanosoma*, *Plasmodium* and *Leishmania*) (Table S8) a very promising activity of **1** against *Leishmania donovani*, the causative agent of visceral leishmaniasis, was observed ($IC_{50}$ 0.5 µM) that was comparable to the positive control miltefosine ($IC_{50}$ 0.3 µM) (Table 1). While **2** showed a 4 times weaker activity compared to **1**, all other compounds were even less potent pointing to a specific target of **1** in *L. donovani* that requires the 3'-configuration found in **1** and **3** as well as the 1,5-dione moiety. Unfortunately, no activity against *Leishmania* infected macrophages could be detected indicating that isatropolones do not reach the intracellular pathogen.

Since the structures of the isatropolones suggested an biosynthesis related to that of the rubrolones, the genome of strain Gö66 was sequenced allowing the comparison of the rubrolone and isatropolone producing BGCs. Antismash analysis [14] of the Gö66 genome sequence led to the identification of two BGCs encoding type II PKS. One of them encoded

an extra ketosynthase III that was assumed to be involved in the biosynthesis of the butyryl starter unit as described in the biosynthesis of R1128,[15] alnumycin [16] and rubrolone [12] to which the genes have the highest similarity (Fig. 1 and Fig. S4a, Table S9). Heterologous expression of *istG-R* in *S. lividans* (Fig. S5) resulted in a strongly red pigmented phenotype as also observed for Gö66 (Fig. S6). HPLC/MS analysis of extracted agar plates revealed the presence of compounds **9**-**10** (Fig. S7, Table S1). Structure elucidation of **9** revealed the presence of a reduced tropolone derivative as a mixture of two diastereomers (Table S10, Fig. 2). Additionally, the expected oxidized aglycon **10** was also detected by its MS and UV data (Fig. S7) as described previously,[12] both confirming the involvement of the *ist* BGC in isatropolone biosynthesis very similar to the proposed rubrolone biosynthesis pathway (Fig. S8). The rearrangement during the tropolone ring formation is not only supported by the occurrence of the required genes in the gene cluster (Table S9) but also from in depth labelling experiments showing a $^{2}J_{CC}$ coupling between C-11 and C-12 after feeding of [1,2-$^{13}C_2$]acetate (Fig. 1, Tables S2-S4).

Interestingly, the *ist* BGC is different to the recently described *rub* BCG [12] as it shows no sugar biosynthesis genes and no acyl carrier protein (ACP) involved in starter unit biosynthesis (Fig. S4a). However, sugar biosynthesis genes resembling those of rubrolone biosynthesis have been found in the genome but 290 kb away. Nevertheless, production of **9** and **10** from expression of *istG-R* in *S. lividans* revealed that the ACP might come from the *S. lividans* fatty acid biosynthesis pathway because no suitable sugar is available for glycosylation in *S. lividans* thus leading to production of the aglycon only (Fig. S4b). While it was not clear in the rubrolone biosynthesis which of the three oxygenases are involved in tropolone ring formation,[12] production of the tropolone derivatives **9** and **10** after expression of *istG-R* indicates that IstP (=RubB) seems to be the only oxygenase required

for this rearrangement. Additionally, the dehydrogenase IstL, the reductase IstO and the dehydratase IstQ might also be involved.

Besides its rare tropolone biosynthesis, being a novel variation of the well-known type II PKS biosynthesis pathway, the ability of isatropolones to bind amines is another unusual feature. Several different amines showed reactivity with **1** but especially lysine showed a very efficient conversion (Fig. 3a and Fig. S8, Table S11) that was also observed for the aglycon **10** (Fig. S9). Not surprisingly, the UV (Fig. S10a) and fluorescence spectra (Fig. 3b and Fig. S10b) of **1** and **6** are quite different allowing the simple identification of isarubrulones even when bound to proteins and thus allowing the labelling of lysine containing proteins as exemplified for bovine serum albumin (BSA) (Fig. 3b, Fig. S11). Reaction of different lysine-containing peptides (Fig. 4a, Fig. S12a) with **1** followed by MS analysis resulted in the expected shift of these peptides by 438 Da resulting from the generated isarubrolone modification (Fig. 4b, Fig. S12b).

The reactivity of the 1,5-dione containing isatropolones towards amines including lysine residues of proteins might be the reason for their promising biological activity against *L. donovani.* Although **1** showed no activity against intracellular *Leishmania* it would be interesting to elucidate its molecular target in this pathogen that might allow the future development of anti-*Leishmania* drugs. Since **1** is available from two companies[17] such research is also open to groups with no access to *Streptomyces* cultivation and natural product isolation. Moreover, the unusual fluorescence properties of isatropolones might motivate the development of similar or simplified 1,5-diones as tools for the covalent labelling and visualization of amine containing molecules including peptides and proteins similar to commercially available dyes (e.g. Cy dyes). Finally, several pyridine containing

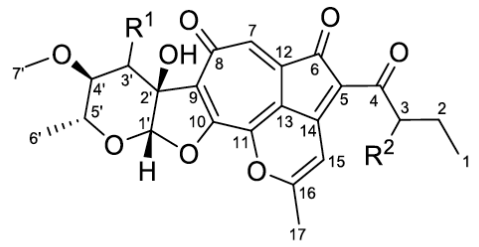
natural products are known that might be derived in a similar reaction starting with a 1,5-dione.[18-23] Although some of these show interesting bioactivities it would be even more interesting to see also their reactivity of the respective 1,5-diones.

**Acknowledgements**

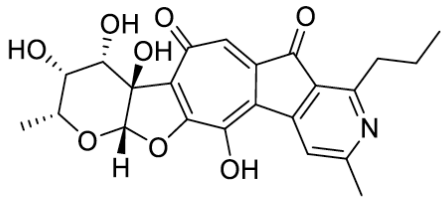
Work in the Bode lab was supported by the DFG and an ERC starting grant (Grant 311477) as well as by the European Community's Seventh Framework Program (FP7/2007–2013) under Grant agreement No. 602773 that also supported M. Kaiser. Y.-M. Shi is the recipient of a Postdoctoral Research Fellowship from the Alexander von Humboldt Foundation. Genome sequencing in the Stinear lab was supported by a National Health and Medical Research Council (NHMRC) of Australian project grant 1105522. Work at BMRZ is supported by the state of Hesse. H. Schwalbe is member of the DFG-cluster of excellence: macromolecular complexes.

## References

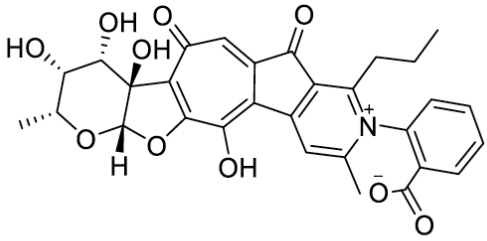

[1] M. Gersch, J. Kreuzer, S. A. Sieber, *Nat Prod Rep* **2012**, *29*, 659–682.
[2] M. H. Wright, S. A. Sieber, *Nat Prod Rep* **2016**, *33*, 681–708.
[3] A. F. Kluge, R. C. Petter, *Current Opinion in Chemical Biology* **2010**, *14*, 421–427.
[4] H. Li, A. J. O'Donoghue, W. A. van der Linden, S. C. Xie, E. Yoo, I. T. Foe, L. Tilley, C. S. Craik, P. C. A. da Fonseca, M. Bogyo, *Nature* **2016**, *530*, 233–236.
[5] E. Génin, M. Reboud-Ravaux, *Curr Top Med Chem* **2010**, *10*, 232–256.
[6] S. Khare, A. S. Nagle, A. Biggart, Y. H. Lai, F. Liang, L. C. Davis, S. W. Barnes, C. J. N. Mathison, E. Myburgh, M.-Y. Gao, et al., *Nature* **2016**, *537*, 229–233.
[7] T. Böttcher, M. Pitscheider, S. A. Sieber, *Angew. Chem. Int. Ed.* **2010**, *49*, 2680–2698.
[8] J.-M. Gao, S.-X. Yang, J.-C. Qin, *Chem. Rev.* **2013**, *113*, 4755–4811.
[9] R. C. Clark, S. Y. Lee, M. Searcey, D. L. Boger, *Nat Prod Rep* **2009**, *26*, 465–13.
[10] W. Schüep, J. F. Blount, T. H. Williams, A. Stempel, *J. Antibiot.* **1978**, *31*, 1226–1232.
[11] Y. Yan, Y.-T. Ma, J. Yang, G. P. Horsman, D. Luo, X. Ji, S.-X. Huang, *Org. Lett.* **2016**, *18*, 1254–1257.
[12] Y. Yan, J. Yang, Z. Yu, M. Yu, Y.-T. Ma, L. Wang, C. Su, J. Luo, G. P. Horsman, S.-X. Huang, *Nature Communications* **2016**, *7*, 1–10.
[13] N. Pöhlmann, *Isolierung, Strukturaufklärung Und Biosynthese Der Isatropolone Aus Streptomyces Sp. Gö66*, Göttingen, **2004**.
[14] K. Blin, M. H. Medema, D. Kazempour, M. A. Fischbach, R. Breitling, E. Takano, T. Weber, *Nucleic Acids Res.* **2013**, *41*, W204–12.
[15] T. Marti, Z. Hu, N. L. Pohl, A. N. Shah, C. Khosla, *J. Biol. Chem.* **2000**, *275*, 33443–33448.
[16] T. Oja, K. Palmu, H. Lehmussola, O. Leppäranta, K. Hännikäinen, J. Niemi, P. Mäntsälä, M. Metsä-Ketelä, *Chem. Biol.* **2008**, *15*, 1046–1057.
[17] Bioviotica Naturstoffe GmbH (http://www.bioviotica.com) and Santa Cruz Biotechnology (https://www.scbt.com).
[18] D. Tasdemir, K. M. Marshall, G. C. Mangalindan, G. P. Concepcion, L. R. Barrows, M. K. Harper, C. M. Ireland, *J. Org. Chem.* **2001**, *66*, 3246–3248.
[19] T. U. Rakhmatullaev, S. Y. Yunusov, *Chemistry of Natural Compounds* **1973**, *9*, 56–58.
[20] S. Oshimi, J. Deguchi, Y. Hirasawa, W. Ekasari, A. Widyawaruyanti, T. S. Wahyuni, N. C. Zaini, O. Shirota, H. Morita, *J. Nat. Prod.* **2009**, *72*, 1899–1901.
[21] C. Dan, Y. Zhou, D. Ye, S. Peng, L. Ding, M. L. Gross, S. X. Qiu, *Org. Lett.* **2007**, *9*, 1813–1816.
[22] L. Jayasinghe, C. P. Jayasooriya, N. Hara, Y. Fujimoto, *Tetrahedron Letters* **2003**, *44*, 8769–8771.
[23] B. Baghdikian, E. Ollivier, R. Faure, L. Debrauwer, P. Rathelot, G. Balansard, *J. Nat. Prod.* **1999**, *62*, 211–213.



Isatropolone A (**1**) $R^1$ = $\beta$-OH; $R^2$ = H
Isatropolone B (**2**) $R^1$ = $\alpha$-OH; $R^2$ = H
Isatropolone C (**3**) $R^1$ = $\beta$-OH; $R^2$ = OH

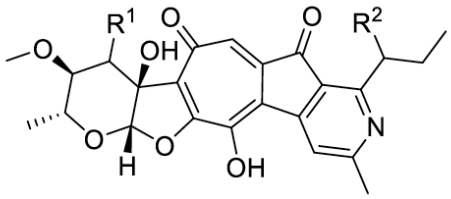


Rubrulone A (**4**)



Rubrulone B (**5**)

((Structures of Isatropolones A-C (**1**-**3**) and Rubrolones A (**4**) and B (**5**)))



Isarubrolone A (**6**) $R^1$ = $\beta$-OH; $R^2$ = H
Isarubrolone B (**7**) $R^1$ = $\alpha$-OH; $R^2$ = H
Isarubrolone C (**8**) $R^1$ = $\beta$-OH; $R^2$ = OH

((Structures of Isarubrolones A-C (**6**-**8**)))

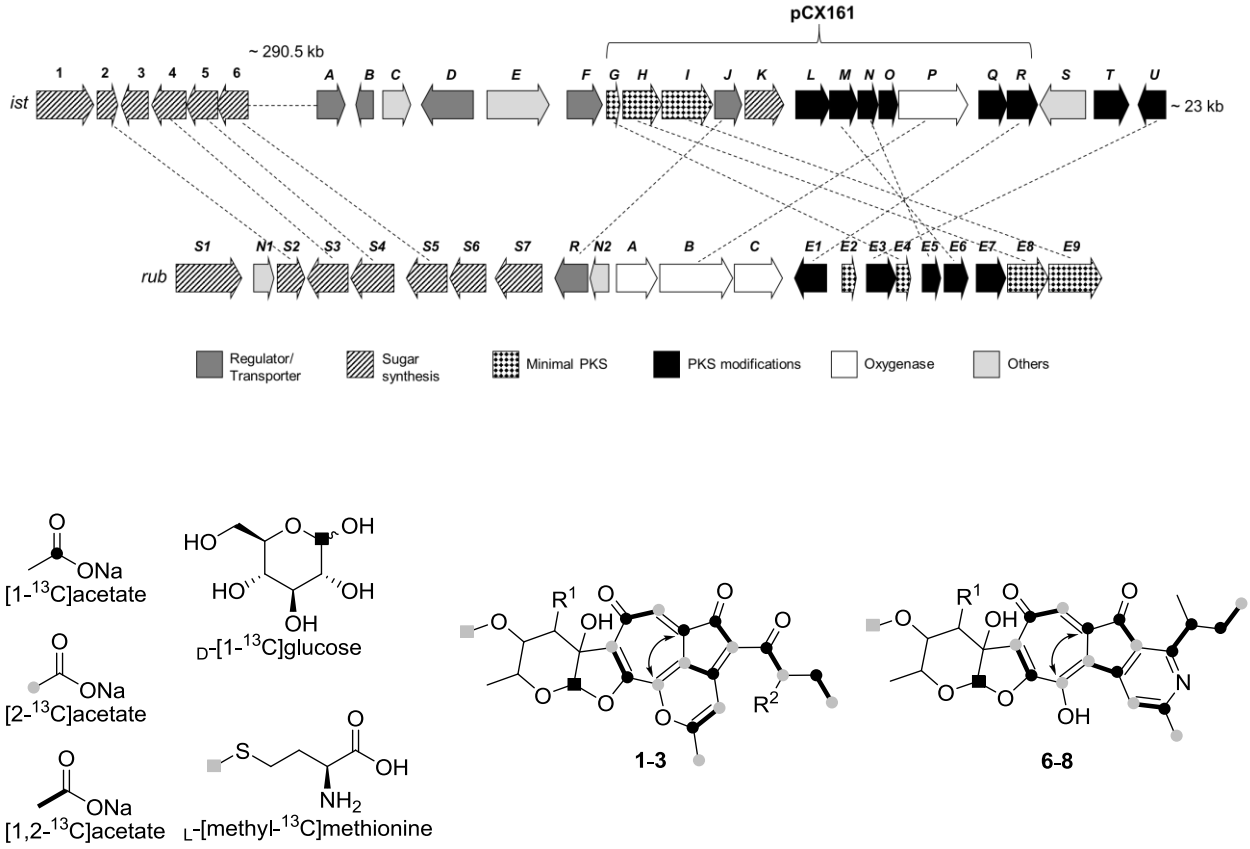



**Figure 1.** Schematic representation of the isatropolone biosynthesis gene cluster (*ist*) in comparison with rubrolone biosynthesis gene cluster (*rub*). The expression plasmid pCX161 encoded *istG-R*, similarities between *ist* and *rub* genes are indicated (top) Labeling pattern of **1**–**6** by [1-$^{13}$C]acetate, [2-$^{13}$C]acetate, [1,2-$^{13}C_2$]acetate, L-[methyl-$^{13}$C]methionine, and D-[1-$^{13}$C]glucose (arrow representing the existence of a small $^2J_{CC}$ value of 0.0-3.3 Hz between C-11 and C-12) (bottom).

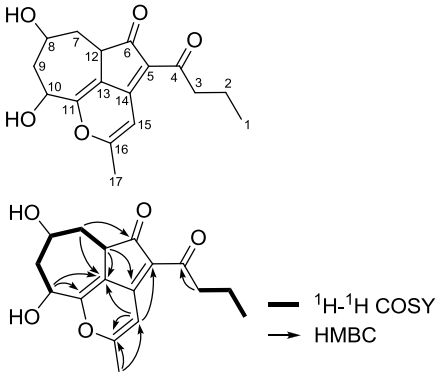



**Figure 2.** Key HMBC (arrows) and $^{1}H$-$^{1}H$ COSY (bold) correlations of reduced intermediate **9**. For more data see Table S10.

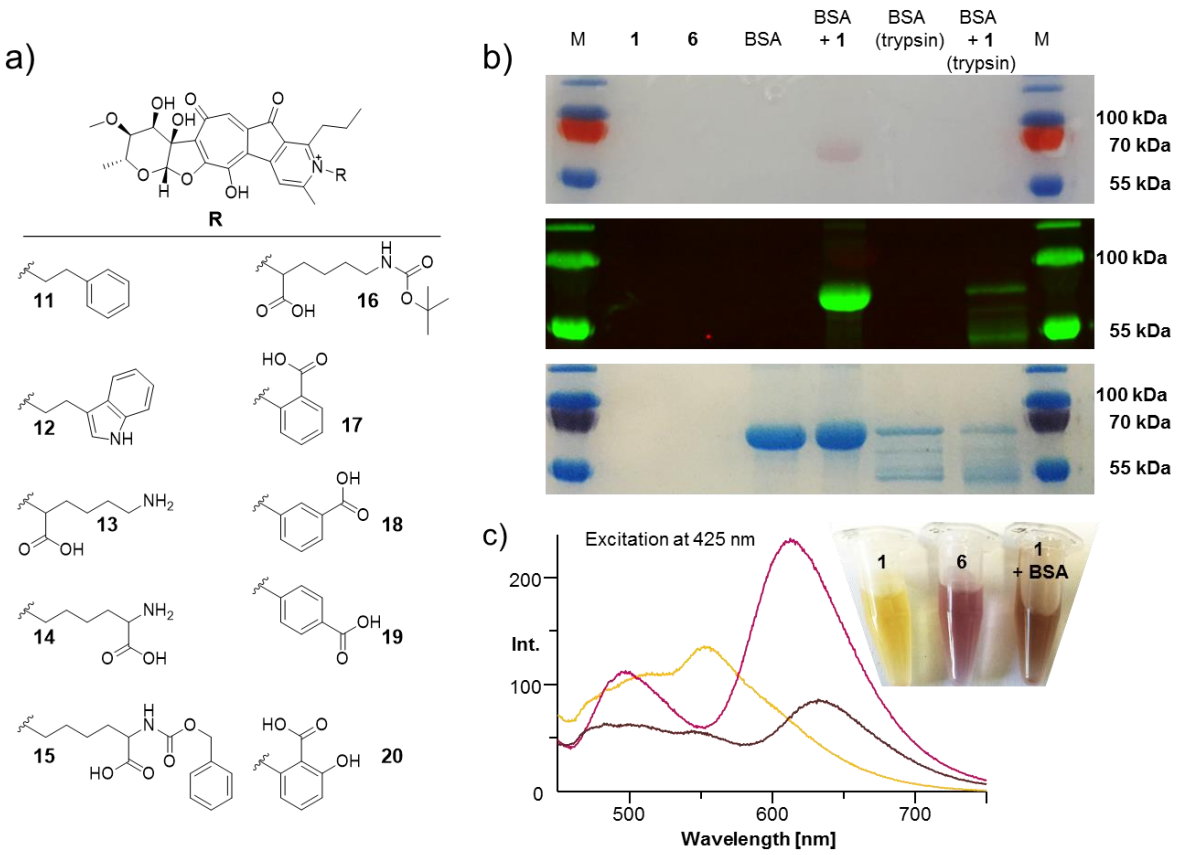



**Figure 3.** a) Chemical structures derived from **1** reacted with amines and amino acids; for detailed HPLC/MS see Fig. S9 and Table S11 for conversion rates, b) SDS-PAGE showing the labelling of bovine serum albumin (BSA) with **1**. Top (white light), middle (fluorescence at 695 nm after excitation at 590-660 nm), bottom (coomassie staining). M, Prestained protein ladder (see Fig. S12 for the full size gel pictures). A time limited trypsin digest of BSA was additionally analyzed. c) Fluorescence spectra of **1**, **6** and **1** reacted with BSA with excitation at 425 nm. For UV chromatograms of **1**, **6** and **1** reacted with BSA and the other fluorescence spectra at different wavelength, see Fig. S11.

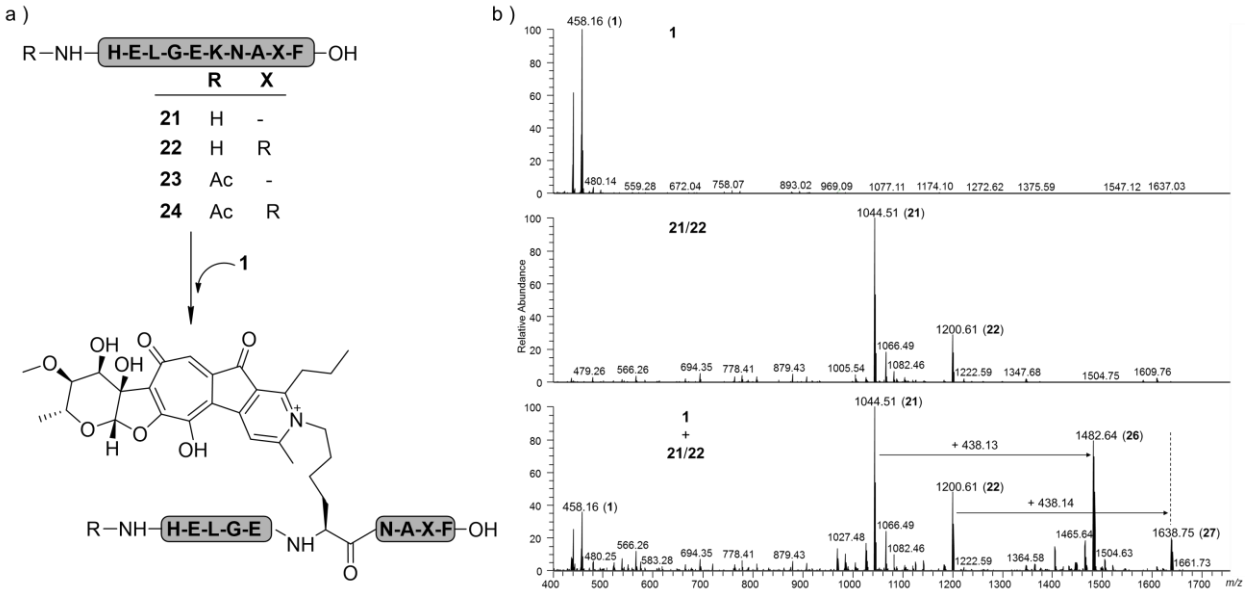



**Figure 4.** Schematic overview of lysine-labelling of synthetic peptides **21**-**24** with **1** (**a**) and corresponding MALDI-MS data of **21**/**22** with and without **1** (**b**).

**Table 1.** Bioactivity of isatropolone A-C (**1**-**3**), isarubrolone A-C (**6-8**) and reduced aglycone **9** against *Leishmania donovani* (visceral leishmaniasis) and rat skeletal myoblasts (L6 cells, cytotoxicity). Miltefosine and podophyllotoxin were used as positive controls.

| Compound | $IC_{50}$ (μM) | |
|---|---|---|
| | ***L. donovani*** | **L6 cells** |
| **1** | 0.5 | 89.8 |
| **2** | 94.6 | >200 |
| **3** | 2.3 | 96.3 |
| **6** | 8.0 | 108.2 |
| **7** | 52.5 | >200 |
| **8** | 18.3 | 92.90 |
| **9** | 120.3 | 177.6 |
| miltefosine | 0.3 | – |
| podophyllotoxin | – | $9.6 \times 10^{-3}$ |