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Rigorózní práce

Totální syntéza [$^{15}N_4$] cytokininů a jejich využití

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Annotation

Cytokinins (CKs) and their metabolites and derivatives are essential for cell division, plant growth regulation and development. They are typically found at minute concentrations in plant tissues containing very complicated biological matrices. Therefore, defined standards labelled with stable isotopes are required for precise metabolic profiling and quantification of CKs, as well as in vivo elucidation of CK biosynthesis in various plant species. In this work, eleven [^{15}N]-labelled C^6-purine derivatives were prepared, among them five aromatic (**4**, **5**, **6**, **7**, **8**) and three isoprenoid (**9**, **10**, **11**) CKs. Compared to current methods, optimized syntheses of 6-amino-9*H*-[$^{15}N_5$]-purine (adenine, **1**) and 6-chloro-9*H*-[$^{15}N_4$]-purine (6-chloropurine, **3**) were performed to achieve more effective, selective and generally easier approaches. The chemical identity and purity of prepared compounds were confirmed by physico-chemical analyses (TLC; HRMS; HPLC–MS; ^{1}H, ^{13}C, ^{15}N NMR). The presented approach is applicable for the synthesis of any other desired [$^{15}N_4$]-labelled C^6-substituted purine derivatives.

Prohlašuji, že svoji rigorózní práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury. Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své rigorózní práce, to v nezkrácené podobě elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

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Prohlášení o autorském podílu

Prohlašuji, že zde předložená rigorózní práce byla vypracována na základě publikace přiložené v příloze 1. Publikace byla publikovaná vědeckým časopisem s impakt faktorem přesahujícím 0.3. Konkrétně 2.515 v roce vydání publikace a 2.924 v pětiletém průměru.

Dále čestně prohlašuji, že na tvorbě publikace jsem se podílel významnou mírou, konkrétně:

- Optimalizací jednotlivých kroků syntetického řetězce od vstupní anorganické molekuly až po všechny prezentované [$^{15}N_4$] C^6-substituované deriváty purinu.
- Charakterizací meziproduktů a produktů syntézy pomocí kapalinové chromatografie s detekcí diodovým polem a následnou hmotnostní detekcí tandemovým hmotnostním spektrometrem (UHPLC-DAD-MS/MS).
- Charakterizací meziproduktů a produktů syntézy pomocí nukleární magnetické rezonance (^{1}H, ^{13}C NMR).
- Samostatným sepsáním výsledků do formy vhodné k publikaci.

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Poděkování

Rád bych poděkoval všem spoluautorům publikace, jejichž rady a vědomosti z dlouhodobé vědecké praxe byly velmi užitečné v průběhu celého procesu vzniku publikace. Konkrétně pak RNDr. Marku Zatloukalovi, Ph.D. a Mgr. Karlu Doležalovi, Dr. za skvělé vedení.

Obsah

1. Úvod

Cytokininy, rostlinné hormony, jsou organické molekuly aktivní již při velmi malých koncentracích (pmol.g^{-1} čerstvé váhy). Z fyziologického hlediska ovlivňují řadu procesů v průběhu růstu a vývoje rostliny. Esenciální roli hrají při buněčném dělení (zde v součinnosti s auxiny), stimulaci fotosyntetických procesů, zpomalování stárnutí rostlinných tkání a v neposlední řadě zvyšují resistenci proti stresu (Mok a Mok, 2001; Miller *et al.*, 1955; Kamínek, 2015).

Z chemického hlediska se jedná o N^6-substituované deriváty adeninu. Na základě struktury postranního řetězce se dále dělí na isoprenoidní, reprezentované isopentenyladeninem, *trans*- a *cis*-zeatinem, a aromatické, ke kterým řadíme benzylaminopurine, *ortho*-, *meta*- a *para*-topolin (Aremu *et al.*, 2012; Strnad *et al.*, 1997). V rostlinných buňkách se cytokininy vyskytují ve formě volných bází, ribosidů, nukleotidů, *O*-glukosidů a *N*-glukosidů (Werner a Schmülling, 2009).

Biologická aktivita cytokininů je závislá nejen na jejich koncentraci, ale také na společném poměru s ostatními rostlinnými hormony. Důležitá je i výše zmíněná chemická struktura. Zatímco volné báze a ribosidy vykazují vysokou biologickou aktivitu, *N*-glukosidy a *O*-glukosidy jsou buď zcela biologicky inaktivní nebo fungují jako zásobní formy (Werner a Schmülling, 2009). V případě isoprenoidních cytokininů hraje roli struktura postranního řetězce. Je dokázáno, že *trans*-izomery vykazují obecně vyšší biologickou aktivitu než *cis*-izomery (Strnad *et al.*, 1997; Mok a Mok, 2001).

V posledních letech byla připravena celá řada nových syntetických cytokininů a jejich derivátů se zcela novou biologickou aktivitou (Mik *et al.*, 2011; Zatloukal *et al.*, 2008). Detailně byla popsaná například silná cytotoxická aktivita vůči lidským nádorovým buňkám (Doležal *et al.*, 2006; Hajduch *et al.*, 1997) nebo pozitivní efekt na lidské fibroblasty, což vede k potenciálním aplikacím na poli kosmetiky (Szüčová *et al.*, 2009).

Jak bylo zmíněno, koncentrace cytokininů a jejich derivátů je klíčová pro jejich biologickou aktivitu. Při biologických aplikacích mimo rostlinnou říši je zároveň kritické determinovat, zda nejen výchozí molekula, ale také některý z jejích metabolitů nevykazuje za určitých koncentrací nežádoucí cytotoxicitu. Z těchto důvodů je zcela nezbytné vyvinout nástroje pro velmi citlivou a přesnou endogenní kvantifikaci a metabolické profilování napříč biologickými systémy.

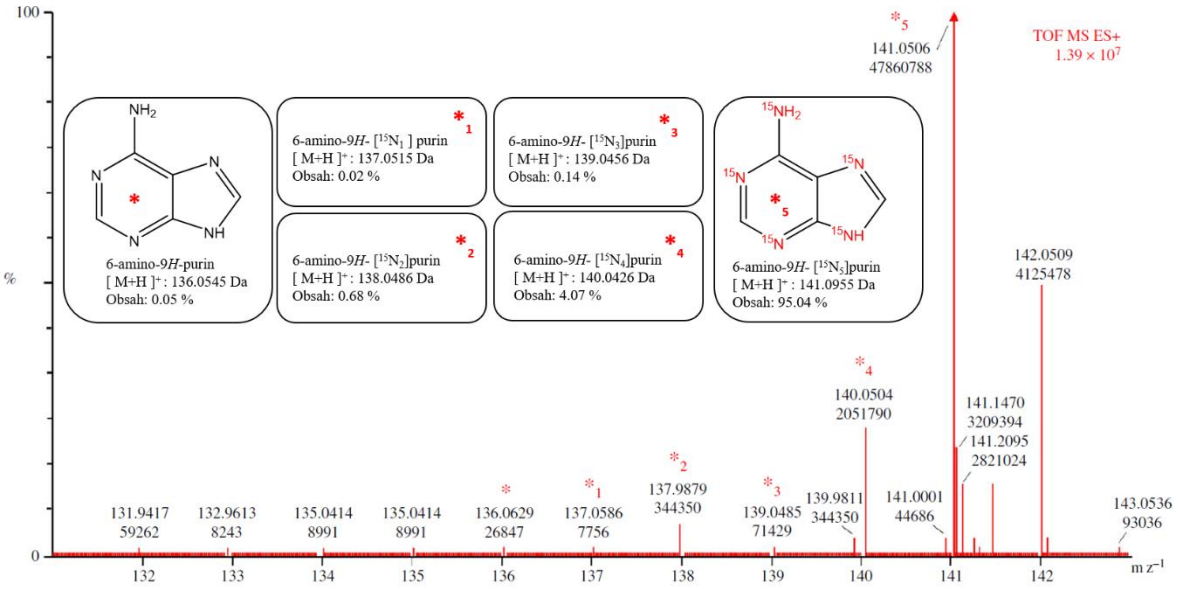
Analýza cytokininů v komplexních biologických matricích je složitá díky jejich velmi nízké koncentraci (pmol.g^{-1} čerstvé váhy) a silnému matričnímu efektu. Metody stanovení cytokininů se obecně skládají z pre-koncentrace a purifikace vzorku s následnou analytickou koncovkou. Tou je v dnešní době nejčastěji vysokoúčinná kapalinová chromatografie s tandemovou hmotnostní detekcí (UHPLC-MS/MS) s využitím techniky izotopového zřeďování (Antoniadi *et al.*, 2015; Novák *et al.*, 2008). Metoda izotopového zřeďování je založena na adici známého množství izotopově značených cytokininů do směsi s neznámým množstvím endogenních (neznačených) cytokininů. Výsledná determinace množství endogenních molekul je realizována skrze porovnání intenzit signálů. Izotopově značné molekuly jsou v hmotnostním spektru jednoduše rozpoznatelné díky rozdílné molekulové hmotě. Důležité je zmínit, že fyzikálně-chemické vlastnosti izotopově značených látek jsou vůči neznačeným zcela identické. Mimo jiné lze izotopově značené deriváty cytokininů použít jako interní standardy korigující iontovou supresi při kvantifikaci pomocí hmotnostní spektrometrie (MS). V neposlední řadě také pro kontrolu specificity, selektivity a návratnosti nově vyvíjených analytických metod. K dnešnímu dni přispěly izotopově značené cytokininy k celé řadě studií zaměřených na rostlinnou fyziologii (Strik *et al.*, 2011; Aremu *et al.*, 2014), profilování cytokininů (Novák *et al.*, 2008; Antoniady *et al.*, 2015; Aremu *et al.*, 2014; Yokoya *et al.*, 2010), mezidruhové interakce (Krall *et al.*, 2002; Siddique *et al.*, 2015) a cytokininový metabolismus a biosyntézu (Kakimoto, 2001; Lindner *et al.*, 2014). Ve všech případech autoři použili deuterované standardy.

Syntéza neznačených cytokininových standardů je k dnešnímu dni velmi dobře popsaná a funkční (Mik *et al.*, 2011; Plíhalová *et al.*, 2016; Tolman *et al.*, 1999). Naopak postupy využívající značené molekuly cytokininů jsou často nekompletní a s velkým prostorem pro optimalizaci (viz kapitola 3. Syntéza). Ve většině případů pak autoři používají ke značení nuklidy vodíku a uhlíku (^{2}H, ^{3}H, ^{13}C). Jestliže vypustíme radioaktivní ^{3}H z důvodů diskomfortu a nebezpečí při práci, tak ani ^{2}H není ideálním nuklidem především kvůli rozdílným fyzikálně-chemickým vlastnostem oproti přirozenému nuklidu vodíku a možné hydrogen-deuteriové výměně v průběhu manipulace (Kushner *et al.*, 1999). Přihlédneme-li ke struktuře purinového jádra (obsahuje čtyři přirozeně se vyskytující nuklidy dusíku, $^{14}N_4$) je volba kompletní substituce těchto nuklidů za stabilní izotopy ^{15}N zcela logická. Přestože postupy popisující dílčí syntézu [$^{15}N_5$]-adeninu, [$^{15}N_4$]-*trans*-zeatinu či cyklizaci formamidu na adenin existují (Horgan *et al.*, 1980; Laxer *et al.*, 2001), kompletní několikastupňová

optimalizovaná syntéza z [^{15}N]-formamidu na koncové [$^{15}N_4$]-aromatické a isoprenoidní cytokininy je zde popsána vůbec poprvé.

2. Determinace izotopové čistoty

Izotopová čistota, tedy obsah jednotlivých izotopologů [$^{15}N_5$], [$^{15}N_4$], [$^{15}N_3$], [$^{15}N_2$], [$^{15}N_1$] a [$^{14}N_5$], byla určena pro každou připravenou molekulu na základě HRMS analýzy. Majoritní podíl [$^{15}N_5$] izotopologu a naopak minimální podíl neznačené molekuly je pro prezentovaný přístup kritický. Metodika výpočtu je demonstrována na obrázku č.1. V tomto případě pro 6-amino-9*H*-[$^{15}N_5$]-purine ([$^{15}N_5$]-adenine, 1). Aplikována byla však pro všechny zde prezentované molekuly.



Obrázek č. 1 – Celkové spektrum z vysokorozlišovací hmotnostní analýzy (HRMS) 6-amino-9*H*-[$^{15}N_5$]-purinu obsahující všechny izotopology. Informace o procentuálním obsahu každého z nich je uvedena v příslušném boxu.

3. Syntéza

Zde prezentovaný postup přípravy [$^{15}N_4$]-značených cytokininů lze obecně rozdělit na dvě syntetické větve popisující přípravu osmi koncových produktů, konkrétně pěti aromatických (**4** - **8**), respektive tří isoprenoidních cytokininů (**9** - **11**). Společné meziprodukty a výchozí molekuly jsou označeny pod čísly **1** - **3**. Konkrétní optimalizace vzhledem k již známým postupům jsou uvedeny v sekci každé připravené molekuly. Kompletní reakční schéma je uvedeno níže (Schéma č. 1).

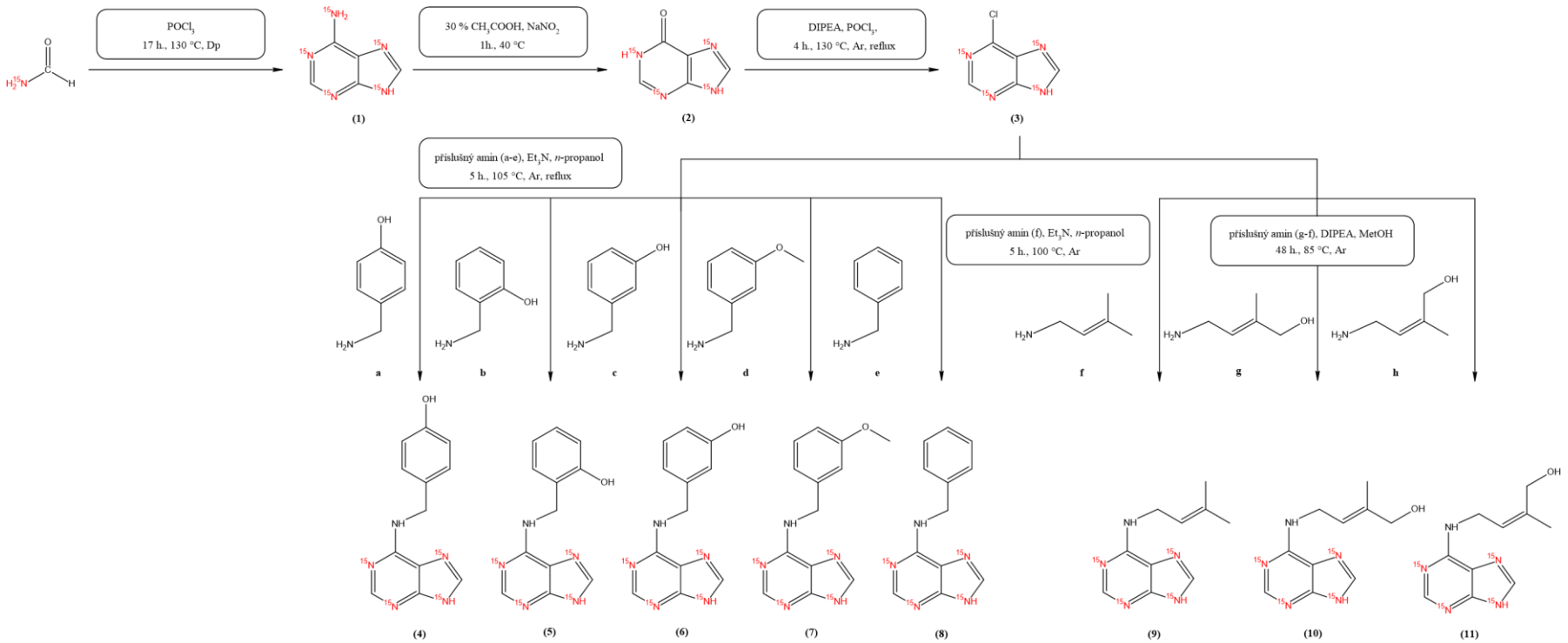


Schéma č. 1 - Reakční schéma syntézy [$^{15}N_4$]-cytokininů. Podmínky reakce mezi jednotlivými kroky jsou uvedeny v popisu. *(a)* 4-hydroxybenzylamin; *(b)* 2-hydroxybenzylamin; *(c)* 3-hydroxybenzylamin; *(d)* 3-methoxybenzylamin; *(e)* benzylamin; *(f)* 3-metylbut-2-en-1-amin; *(g)* (E)-4-amino-2-metylbut-2-en-1-ol; *(h)* (Z)-4-amino-2-metyl-2-buten-1-ol.

3.1 Syntéza meziproduktů

3.1.1 6-amino-9*H*-[$^{15}N_5$]-purin (adenin) (1)

[$^{15}N_5$]-adenin hraje v případě zde prezentované syntézy naprosto klíčovou roli výchozí molekuly. Jeho příprava s co nejvyšším procentuálním výtěžkem a poměrem isotopologu [$^{15}N_5$] je kritická. Z množství možných syntetických postupů přípravy adeninu se jevila jako nejjednodušší již popsaná termická cyklizace z formamidu za použití $POCl_3$ v poměru 1:2 (Ochiai *et al.*, 1968). Po detailní analýze vysokorozlišovací hmotnostní spektrometrií (HRMS) a NMR (^1H, ^{13}C) však vyšla najevo přítomnost vedlejšího produktu, [$^{15}N_5$]-1*H*-imidazo-[4,5-b]-pyrazine-5-aminu, a to v poměru 1:1 k [$^{15}N_5$]-adeninu (Schéma č. 2). Původní autoři tento vedlejší produkt nepopsali především díky absenci analytických metod. Studie publikována o deset let později však jeho přítomnost již zmiňuje stejně tak jako postup eliminace založený na stereo specifické eliminaci na soli pikrátu (Apene *et al.*, 1978). Tento postup se ovšem nepodařilo uspokojivě zreprodukovat.

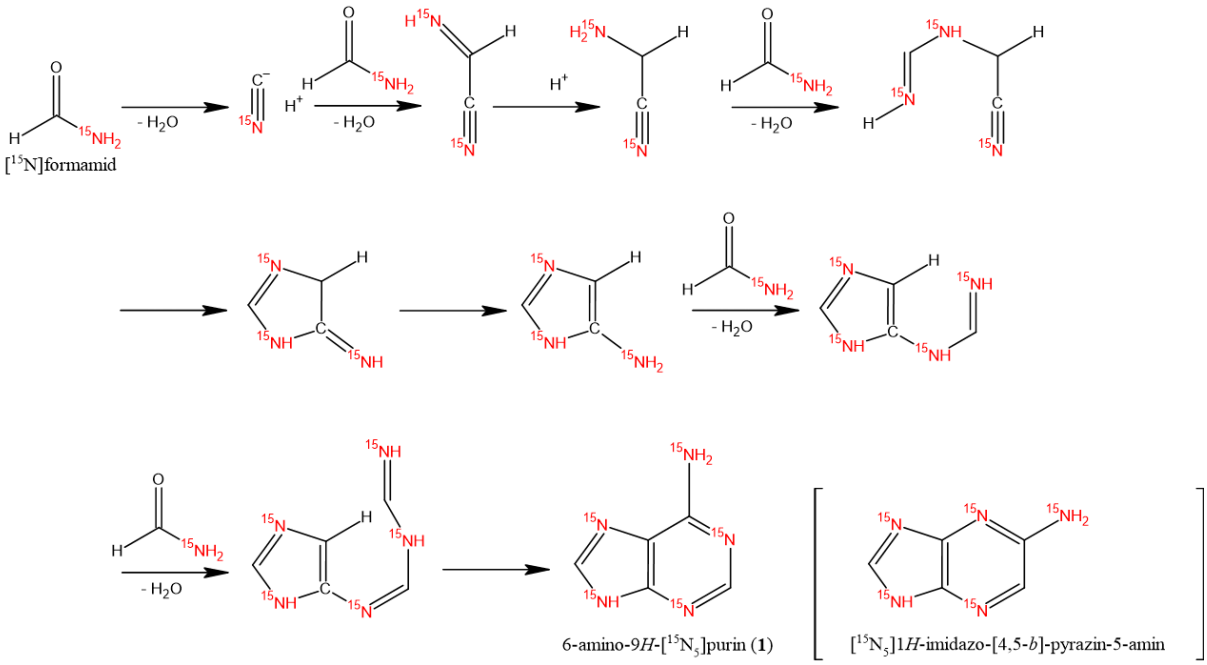
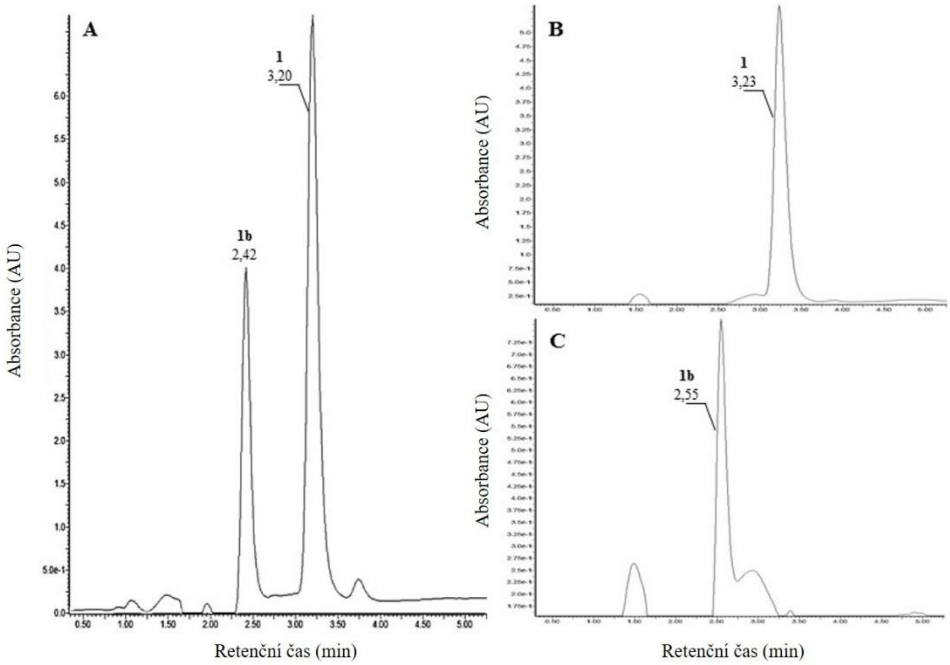


Schéma č. 2 – Předpokládané reakční schéma cyklizace [^{15}N]-formamidu na [$^{15}N_5$]-adenin (**1**) současně za vzniku [$^{15}N_5$]-1*H*-imidazo-[4,5-b]-pyrazine-5-aminu (Hudson *et al.*, 2012).

Z výše popsaného vyplývá nutnost vyvinout vysoce selektivní metodu separace [$^{15}N_5$]-adeninu a [$^{15}N_5$]-1*H*-imidazo-[4,5-b]-pyrazine-5-aminu. Zatímco výchozí podmínky syntézy byly zachovány dle Ochiai *et al.*, 1968, následující purifikační a separační kroky byly významně modifikovány. Reakce byla přesunuta do vysokotlakého reaktoru s polytetrafluorethylenovým (PTFE) insertem. Zbylé $POCl_3$ bylo odstraněno destilací a reakční

směs přenesena do prostředí iontoměniče v kyselé formě (DOWEX 50, H+) na 48 hodin. Tímto byly eliminovány zbylé nezreagované intermediáty z procesu cyklizace. Směs [$^{15}N_5$]-adeninu a [$^{15}N_5$]-1*H*-imidazo-[4,5-b]-pyrazine-5-aminu byla eluována 5M NH_4OH. V posledním purifikačním kroku byla k rozdělení zmíněných produktů nasazena kolonová chromatografie za použití chloroformu/metanolu/amoniaku v poměru 6:1:0.05 jako mobilní fáze. [$^{15}N_5$]-adenin byl takto izolován s výtěžkem 32 %, v 98% chromatografické čistotě (HPLC-DAD) a izotopové čistotě 95 % pro [$^{15}N_5$] izotopolog.

Jednotlivé frakce obsahující [$^{15}N_5$]-adenin a [$^{15}N_5$]-1*H*-imidazo-[4,5-b]-pyrazine-5-amin byly podrobeny HPLC-DAD a HPLC-DAD-MS pro potvrzení identity. Chromatogramy před a po separaci jsou zobrazeny na obrázku č. 2.



Obrázek č. 2 – HPLC-DAD chromatogramy [$^{15}N_5$]-adeninu (1) a [$^{15}N_5$]-1*H*-imidazo-[4,5-b]-pyrazine-5-aminu (1b) před, respektive po rozdělení za pomocí kolonové chromatografie.

3.1.2 1,7-dihydro-6*H*-[$^{15}N_4$]-purin-6-one (hypoxantin) (2)

Syntéza [$^{15}N_4$]-hypoxantinu je založena na jednoduché deaminaci [$^{15}N_5$]-adeninu ve slabě kyselém prostředí za účasti solí kyseliny dusičné, V tomto případě nebylo nutno nijak optimalizovat podmínky v zavedené literatuře (Kruger *et al.*, 1983).

3.1.3 6-chloro-9*H*-[$^{15}N_4$]-purin (6-chloropurin) (3)

Společně se zmíněným [$^{15}N_5$]-adeninem je [$^{15}N_4$]-6-chloropurin druhá kritická molekula zde prezentovaného postupu. Jakožto společný akceptor isoprenoidního, respektive aromatického řetězce, jeho fyzikálně-chemické vlastnosti přímo determinují vlastnosti cílových produktů. Z tohoto důvodu byla vyvinuta extrémní snaha o optimalizaci reakčního kroku za účelem dosažení co nejvyššího výtěžku, chromatografické a izotopové čistoty.

Obecně je reakce založena na chloraci hypoxantinu v prostředí báze a donoru chlóru. Zatímco donorem je téměř vždy $POCl_3$, volba vhodné báze, reakční teploty a času je předmětem optimalizace. V literatuře jsou popsány postupy využívající *N*,*N*-dimetylanilin (DMA) (Bendich *et al.*, 1954; Taddei *et al.*, 2004), zřídka také postupy zcela bez báze (Sariri *et al.*, 2002).

V průběhu optimalizace byl posledně zmíněný postup první volbou díky své instrumentální nenáročnosti a relativně vysokým výtěžkům (53 %). Reprodukce na [$^{15}N_4$]-hypoxantinu vedla opakovaně k velmi slibným chromatografickým čistotám (až 97 %), ovšem neuspokojivým výtěžkům okolo 10 %. Následné pokusy vycházely z podmínek popsaných v literatuře (Horgan *et al.*, 1980; Bendych *et al.*, 1954). Modifikována byla použitá báze (DMA byl nahrazen trietylaminem, TEA), reakční teplota (původní pokojová teplota byla zvýšena na 105 °C) a konečně reakční doba, která byla díky změně teploty zkrácena z 24 hodin na 4 hodiny. [$^{15}N_4$]-6-chloropurin byl následně z reakční směsi extrahován procesem kontinuální extrakce za použití směsi etylacetátu a vody v poměru 1:1. Výsledný produkt byl takto extrahován s výtěžkem 14 % vzhledem k výchozí látce. Chromatografická čistota dosahovala 85 %. Z důvodu zvýšení výtěžnosti byla doba kontinuální extrakce prodloužena na 48 hodin. Dle očekávání se výtěžek zvedl až na 26 %, chromatografická čistota pak na 90 %. Vezmeme-li v úvahu dobu trvání celé reakce (víc jak 48 hodin), instrumentální náročnost vycházející z kontinuální extrakce a zmíněné výsledky, není tento postup stále vhodný pro rutinní a robustní přípravu finálního meziproduktu. Z toho důvodu byla soustředěna veškerá pozornost na vývoj instrumentálně jednoduché a efektivní reakce.

Původní podmínky ve smyslu reakční teploty a času byly zachovány (105 - 130 °C, 4 hodiny). Jako báze byl nejprve vyzkoušen 2,6-dimetylpyridin, ovšem bez úspěchu. Díky své rozsáhlé aplikaci byla další volbou *N*,*N*-diisopropyletylamin (DIPEA). Reakční směs v poměru [$^{15}N_4$]-hypoxantin:$POCl_3$:DIPEA (1:6:2) byla zpracována za definovaných reakčních podmínek. Produkt byl následně z reakční směsi extrahován diskontinuálně do směsi *tert*-butyl metyl ether:voda (1:1). Výsledná výtěžnost tak dosáhla 95 %. Chromatografická čistota takto

připraveného [$^{15}N_4$]-6-chloropurinu byla stanovena na 97 %. Výše zmíněný postup tak představuje velmi jednoduchou a efektivní cestu chlorace [$^{15}N_4$]-hypoxantinu na [$^{15}N_4$]-6-chloropurin za méně než 8 hodin. Výsledky optimalizace tohoto reakčního kroku včetně reakčních a purifikačních podmínek jsou shrnuty v tabulce č. 1.

Tabulka č. 1 – Sumarizace optimalizace reakce chlorace [$^{15}N_4$]-hypoxantinu (**2**) na [$^{15}N_4$]-6-chloropurin (**3**) včetně detailních reakčních podmínek, purifikačního postupu, výtěžku a chromatografické čistoty. Konečné optimalizované podmínky jsou zvýrazněny tučně.

(1) **(2)**

Reaktanty*	Reakční podmínky		Purifikační postup	Výtěžek [%]	Chromatografická čistota [%]
	Teplota [°C]	Čas [h]			
Acetonitril, Ethylbenzen	65	6	*a*	n.d.	n.d.
Acetonitril, Ethylbenzen	65 - 80	6	*a*	n.d.	n.d.
Acetonitril, Ethylbenzen	100	6	*a*	<10	97
Triethylamin	105	4	*b*	14	85
Triethylamin	105	4	*c*	25	<90
2,6-dimethylpyridin	130	4	*a*	n.d.	n.d.
***N,N*-diisopropylethylamin**	**130**	**4**	***d***	**95**	**97**

* krom zmíněných obsažen vždy [$^{15}N_4$]-hypoxantin a $POCl_3$
a - žádný
b - diskontinuální extrakce do směsi ethylacetát:H_2O (1:1)
c - kontinuální extrakce diethyletherem (48 hod)
d -diskontinuální extrakce do směsi *tert*-butyl metyl ether:H_2O (1:1)

3.2 Syntéza [$^{15}N_4$]-aromatických cytokininů

Jelikož příprava kritických molekul reakčního mechanismu byla optimalizována, syntéza konečných produktů tak byla prováděna s respektem k literatuře bez zásadnějších modifikací.

Proces syntézy [$^{15}N_4$]-aromatických cytokininů je obecně založen na reakci [$^{15}N_4$]-6-chloropurinu s příslušným aminem v prostředí báze (TEA) v poměru 1:1:2. Reakční podmínky byly zcela převzaty z literatury popisující přípravu mono hydroxylovaných derivátů na pozici *para*-, *ortho*- a *meta*- (**4**, **5**, **6**) (Leonard *et al.*, 1975), respektive 6-(3-methoxybenzylamino)-9*H*-[$^{15}N_4$]-purinu (meta-methoxy topolin, **7**)

a 6-benzylamino-9*H*-[$^{15}N_4$]-purinu (BAP, **8**) (Tarkowská *et al.*, 2003; Daly *et al.*, 1956). Výsledný výtěžek respektoval ve všech případech literaturu, chromatografická čistota byla u všech připravených [$^{15}N_4$]-aromatických cytokininů nad 98 %.

3.3 Syntéza [$^{15}N_4$]-isoprenoidních cytokininů

Podobně jako v případě [$^{15}N_4$]-aromatických cytokininů, tak i isoprenoidní analogy byly připraveny dle podmínek uvedených v literatuře, pouze s minoritními změnami.

6-[(3-metylbut-2-en-1-yl)]-9*H*-[$^{15}N_4$]-purin-6-amin (isopentenyl adenine, **9**) byl syntetizován zcela dle literatury s výtěžkem 40 % a chromatografickou čistotou 99 % (Mik *et al.*, 2011). Syntéza 6-[(*E*)-4-hydroxy-3-metylbut-2-en-1-yl]-9*H*-[$^{15}N_4$]-purin-6-aminu (*trans*-zeatin, **10**) a 6-[(*Z*)-4-hydroxy-3-metylbut-2-en-1-yl]-9*H*-[$^{15}N_4$]-purin-6-aminu (*cis*-zeatin, **11**) byla mírně modifikována vzhledem k původní literatuře (Tolman *et al.*, 1999). Vstupní reaktanty byly zachovány, avšak TEA byl nahrazen DIPEA, reakční doba byla prodloužena z původních 5 hodin na 48 hodin a teplota snížena na 85 °C. Výtěžky všech syntetizovaných [$^{15}N_4$]-isoprenoidních cytokininů přesahovaly 40 % s chromatografickou čistotou nad 97 %.

4. Výsledky a diskuse

Práce popisuje přípravu celkem jedenácti [^{15}N] značených derivátů purinu, z toho tří intermediátů (**1**, **2**, **3**), čtyř [$^{15}N_4$] aromatických cytokininů (**4**, **5**, **6**, **7**, **8**) a tří [$^{15}N_4$] isoprenoidních cytokininů (**9**, **10**, **11**).

Identita a čistota všech připravených molekul byla řádně specifikována za pomocí chromatografie na tenké vrstvě (TLC), UHPLC-DAD-MS, HRMS a ^{1}H, ^{13}C, ^{15}N NMR. ^{15}N NMR bylo prováděno pouze v případě kritických intermediátů (1, 3) a vybraného koncového produktu k prokázání struktury (**5**). V obou případech výsledky odpovídaly literatuře (Laxer *et al.*, 2001; Sečkářová, 2001). Změřené body tání byly v souladu s literaturou popisující tento experiment na neznačených molekulách (Hecht *et al.*, 1970; Shaw *et al.*, 1966). Izotopová čistota, tedy celkový obsah [$^{15}N_5$] izotopologu vůči ostatním zbylým izotopologům, byla pro [$^{15}N_5$]-adenin stanovena na 95 %. U všech zbylých produktů syntézy je zachována, jelikož purinové jádro, jakožto nositel [^{15}N] nuklidu, zůstává inertní. Obsah neznačeného isotopologu [$^{15}N_0$], respektive [$^{14}N_5$] byl ve všech případech menší než 0.2 %. Kompletní výsledky fyzikálně-chemického testování jsou pro každou molekulu uvedeny v Příloze 1. Výtěžek reakce, chromatografická čistota a výsledek HRMS analýzy pak v tabulce č. 2.

Tabulka č. 2 – Výtěžek, chromatografická čistota a výsledky analýzy vysoko-rozlišovací hmotnostní spektrometrie (HRMS) pro každou připravenou látku.

Látka	Výtěžek [%]	Chromatografická čistota [%]	MS [M+H+]	Vysoko-rozlišovací hmotnostní spektrometrie (HRMS)			
				Změřená hmota [M+H$^+$]	Teoretická hmota [M+H$^+$]	Molekulový vzorec	Přesnost [ppm]
1	32	98 *	140.89	141.0475	141.0475	$C_5H_5{}^{15}N_5$	0.0
2	71	97 **	141.06	141.0345	141.0345	$C_5H_4{}^{15}N_4O$	0.0
3	95	97 ***	158.81	159.0006	159.0006	$C_5H_3{}^{15}N_4Cl$	0.0
4	80	98 **	246.11	246.0924	246.0923	$C_{12}H_{11}N^{15}N_4O$	0.4
5	78	99 **	245.99	246.0923	246.0923	$C_{12}H_{11}N^{15}N_4O$	0.0
6	55	98 **	246.09	246.0925	246.0923	$C_{12}H_{11}N^{15}N_4O$	0.8
7	85	99 **	260.07	260.1080	260.1080	$C_{13}H_{13}N^{15}N_4O$	0.7
8	41	99 **	230.00	230.0984	230.0974	$C_{12}H_{11}N^{15}N_4O$	0.4
9	40	99 **	207.90	208.1132	208.1131	$C_{10}H_{13}N^{15}N_4$	0.5
10	40	97 ****	224.10	224.1082	224.1080	$C_{10}H_{13}N^{15}N_4O$	0.9
11	41	96 ****	224.00	224.1080	224.1080	$C_{10}H_{13}N^{15}N_4O$	0.0

* čištěno kolonovou chromatografií

** krystalizováno z reakční směsi

*** diskontinuální extrakce do směsi tert-butyl methyl ether:H2O (1:1)

**** odpaření reakční směsi a krystalizace z vody

Přestože syntetické postupy pro výrobu zde prezentovaných molekul v neznačené formě existují (Tarkowská *et al.*, 2003; Mik *et al.*, 2011; Tolman *et al.*, 1999; Baker *et al.*, 1989; Ochiai *et al.*, 1968; Kruger, 1893; Bendich *et al.*, 1954), musely být ve většině případů optimalizovány či zcela změněny. Hlavním důvodem byla minimalizace post-reakčních purifikačních kroků snižujících celkový výtěžek reakce a obecné přenesení reakce do minimalistických podmínek (mg až g množství výchozích látek).

Je třeba zmínit, že práce popisující přípravu cytokininů značených stabilními nuklidy [^{2}H] nebo [^{13}C] jsou také známy. Ve většině případu se však jedná o inkorporaci jednoho (Letham *et al.*, 1971; Chen, 1981; Nguyen *et al.*, 1971) maximálně dvou těchto nuklidů (Letham *et al.*, 1971; Chen, 1981), což vede k hmotnostnímu posunu o 1, respektive 2 m/z. Takové vlastnosti jsou však zpochybnitelné pro případné biosyntetické a metabolické studie vyžadující značené interní standardy pro MS (Novák *et al.*, 2008). Také postupy přípravy vícenásobně stabilně značených cytokininů jsou známy. Konkrétně pak syntéza [$^{2}H_{5}$]-*trans*-zeatinu a [$^{2}H_{4}$]-benzylamino purinu. V obou případech je však většina nuklidů [^{2}H] lokalizována na postranním řetězci, který může být v průběhu biosyntézy modifikován. V neposlední řadě je využití [^{2}H] nuklidů diskutováno kvůli svým rozdílným fyzikálně-chemickým vlastnostem oproti přirozenému nuklidu [^{1}H] a možné vodík-deuteriové výměně (Kushner *et al.*, 1999).

Ve výsledku je zde vůbec poprvé prezentována metodika totální syntézy celkem osmi aromatických, respektive isoprenoidních cytokininů vzniklých cyklizací anorganické molekuly [^{15}N]-formamidu. Všechny připravené deriváty purinu nesou čtyři nuklidy [^{15}N] ukotvené na vysoce inertním purinovém jádře, navíc s obsahem [$^{15}N_{4}$] izotopologu nad 95 %. Prezentovaný syntetický postup je velice versatilní a může být aplikován na přípravu jakýchkoliv dalších [$^{15}N_{4}$]-C^{6}-substituovaných purinových derivátů, o čemž svědčí například studie popisující přípravu [$^{15}N_{4}$] glykosidů (Tranová *et al.*, 2018). Své využití již našly také při charakterizaci nově vyvíjené analytické metody založené na imunopurifikaci cytokininů za pomoci magnetických mikročástic (Plačková *et al.*, 2016).

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Total synthesis of [^{15}N]-labelled C6-substituted purines from [^{15}N]-formamide—easy preparation of isotopically labelled cytokinins and derivatives

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Cytokinins (CKs) and their metabolites and derivatives are essential for cell division, plant growth regulation and development. They are typically found at minute concentrations in plant tissues containing very complicated biological matrices. Therefore, defined standards labelled with stable isotopes are required for precise metabolic profiling and quantification of CKs, as well as *in vivo* elucidation of CK biosynthesis in various plant species. In this work, 11 [^{15}N]-labelled C6-purine derivatives were prepared, among them 5 aromatic (**4, 5, 6, 7, 8**) and 3 isoprenoid (**9, 10, 11**) CKs. Compared to current methods, optimized syntheses of 6-amino-9H-[$^{15}N_5$]-purine (adenine) and 6-chloro-9H-[$^{15}N_4$]-purine (6-chloropurine) were performed to achieve more effective, selective and generally easier approaches. The chemical identity and purity of prepared compounds were confirmed by physico-chemical analyses (TLC; HRMS; HPLC–MS; ^{1}H, ^{13}C, ^{15}N NMR). The presented approach is applicable for the synthesis of any other desired [$^{15}N_4$]-labelled C6-substituted purine derivatives.

1. Introduction

Cytokinins (CKs) are naturally occurring substances in plants derived from adenine with either an aromatic (ARCK) or isoprenoid (ISCK) side chain at the N^6-position. CKs can regulate all stages of plant development and directly affect plant growth processes [1].

Both types of naturally occurring CKs were discovered, quantified and characterized [2–6]. Subsequently, newly prepared CKs and their derivatives were synthesized and tested in several studies for various biological activities [7,8]. Some of the derivatives exhibit a strong cytotoxic effect on human cancer cell lines [9,10]. Others contribute to important applications in pharmacology and cosmetics [11]. As apparent from the number of publications dedicated to various biological effects of CKs and their derivatives, development of sensitive and robust analytical methods for monitoring endogenous concentrations of CKs in various biological systems became crucial.

Analysing CKs in complex biological matrices is difficult because they exist in very low concentrations (pmol g^{-1} fresh weight) [12]. Modern analytical procedures for the determination of CKs consist of sample pre-treatment and subsequent instrumental measurement of individual CK metabolites [13]. At present, endogenous CK metabolites are generally quantified by mass spectrometry (MS) using the isotope dilution technique [12]. Isotopically labelled standards are easily distinguished during MS analysis due to their unique masses. The stable isotope dilution method, which involves determining the concentration of a non-labelled (endogenous) compound and comparing it to that of a labelled internal standard, can be very accurate and precise. Therefore, isotopically labelled CK standards are highly beneficial for controlling selectivity, affinity, recovery and capacity of newly developed analytical procedures and for correction of ion suppression effects during MS analysis.

Preparation methods for CK standards containing naturally occurring nuclides are well-established, documented and functional [7,14,15]. On the contrary, for isotopically labelled CKs, existing preparation methods are problematic and need optimization. Several approaches were recently developed. Hydrogen and carbon nuclides (^2H, ^3H, ^{13}C) are most widely used in the fields of phytohormones and plant physiology. However, considering the characteristics of the molecules prepared herein, only preparation methods for CKs labelled with stable isotopes will be described.

Among ARCKs, the ^2H and ^{15}N isotopologues of 6-benzylaminopurine (BAP) have already been prepared. Deuterium can be incorporated with a catalysed hydrogen–deuterium exchange reaction [16]. A reaction between 6-chloropurine and the corresponding isotopically labelled [$^{15}N_1$] amine can be used to achieve a more stable [$^{15}N_1$]-BAP [17,18]. However, the above-mentioned methods can only produce the [$^{15}N_1$] isotopomer, which is insufficient for use as an internal standard for MS.

Considerable efforts were made to prepare an isotopically labelled analogue of *trans*-zeatin (*t*Z) since it was one of the first discovered and frequently occurring ISCKs. However, chemical, spectroscopic and enzymatic evidence suggest that the zeatin molecule exists as *cis*- and *trans*-geometrical isomers [3]. Thus, a stereospecific synthetic approach had to be developed first to produce a particular geometric isomer. Synthesis of the *trans*-isomer was reported in 1966, based on the reaction of the crude amine with 6-methylthiopurine [19]. However, the first reliable stereospecific method for preparing [$^{13}C_8$]-*t*Z was presented 5 years later [20]. Alternatively, ^2H nuclide can be introduced to the *t*Z side chain as well to produce [$^2H_5$]-*t*Z [21]. A few years later, alongside the first attempt to prepare $^{15}N_4$ *trans*-zeatin [22], another complex approach has been developed, which gradually combines side chain catalytic constructions [23]. By using deuterated reduction systems and solvents, isotopically labelled *t*Z side chains can be produced for reaction with 6-chloropurine [23].

Currently published approaches for isotopically labelled isopentenyladenine (iP) preparation are based mainly on side chain isotopic labelling. Introduction of ^{13}C nuclide into the iP structure can proceed via de novo side chain construction using $^{13}CO_2$ as the nuclide donor [24]. Catalytic reduction of a specific nitrile to an amine using $LiAD_4$ with a subsequent reaction of the amine with 6-chloropurine produced twice-deuterated iP at the N^6 position [25].

Isotopically labelled CK standards contributed in part to several recent studies focused on: plant physiology [26,27]; CK profiling [12,13,27,28]; interspecies interactions [29,30]; and, finally, CK biosynthesis and metabolism [31,32]. Interestingly, deuterated CK standards were used in most of the above-mentioned studies.

Although several approaches to producing isotopically labelled CK standards have been published, no functional and complete approach for easy and relatively inexpensive production from a simple precursor exists. Most of the above-mentioned studies used hydrogen nuclides. Without accounting for radioactive ^3H due to its inherent dangers and lack of comfort with its use, even ^2H is a poor

choice for metabolomic applications because its physico-chemical properties are vastly different from the naturally occurring hydrogen isotope [33]. The possibility of hydrogen–deuterium exchange during laboratory and *in vivo* manipulations present another important disadvantage. These and other considerations as well as the purine-core structure make use of the ^{15}N nuclide a reasonable choice. Despite the existence of previous studies describing [$^{15}N_5$] adenine and *trans*-zeatin preparation [22,34] as well as formamide cyclization to adenine [35], a significant improvement of continuous and fully functional synthesis of ^{15}N-core-labelled CKs from [^{15}N]-formamide is presented herein. Furthermore, the yields are strongly improved compared to the less effective procedures described above.

2. Material and methods

2.1. General procedures

2.1.1. Equipment

Analytical thin-layer chromatography (TLC) was performed using silica gel ALUGRAM Xtra SIL G/UV_{254} plates (Macherey-Nagel, Düren, Germany). An ultraviolet (UV) cabinet with adjustable UV lengths 254/364 nm (Camag, Muttenz, Switzerland) was used for detection. The melting points were determined on Büchi Melting Point B-540 apparatus and are uncorrected. High-performance liquid chromatography–UV–diode array–mass spectrometry (HPLC–UV–DAD–MS) experiments were performed using a Waters 2695 separation module linked with a Waters 2996 photodiode array detector (PDA; Waters, Milford, MA, USA), followed by a hybrid quadrupole time-of-flight (Q-TOF) Micro^{TM} mass spectrometer equipped with electrospray ionization interface (Waters MS Technologies, Manchester, UK). High-resolution mass spectrometry (HRMS) was used to determine the elemental composition of prepared compounds. HRMS involved an ultra-performance liquid chromatography–UV–diode array–mass spectrometry (HPLC–UV–DAD/HPLC–MS) experiment using an Acquity UPLC H-Class system (Waters, Milford, MA, USA) followed by a hybrid Q-TOF tandem mass spectrometer Synapt G2-Si equipped with electrospray ionization interface (Waters MS Technologies, Manchester, UK). Data were processed using MassLynx 4.1 software. NMR spectra were measured by a JEOL ECA-500 spectrometer operating at 19°C and 500.16 MHz (^{1}H), 125.77 MHz (^{13}C) and 50.68 MHz (^{15}N), respectively. Samples were prepared by dissolving the compounds in DMSO-d6. Tetramethylsilane (TMS) for ^{1}H and ^{13}C, and (^{15}N)-ammonium for ^{15}N, were used as external standards.

2.1.2. HPLC–UV–DAD and HPLC–MS conditions

Compounds (1 mg) were dissolved in 1 ml of 1% methanol and injected (10 µl) onto a reversed-phase column (Symmetry C18, 5 µm, 150 × 2.1 mm; Waters, Milford, MA, USA) incubated at 25°C. Solvent A was 15 mM ammonium formate adjusted to pH 4.0. Solvent B was methanol. The following binary gradient was used at a flow-rate of 200 µl min^{-1}: 0 min, 10% B; 0–24 min linear gradient to 90% B; 25–34 min isocratic elution of 90% B; 35–45 min linear gradient to 10% B. The flow was introduced to a DAD detector (scanning range 210–400 nm with 1.2 nm resolution) and then to an electrospray source (source temperature 120°C, desolvation temperature 300°C, capillary voltage 3 kV, cone voltage 20 V). Nitrogen was used as the cone gas (50 l h^{-1}) and the desolvation gas (500 l h^{-1}). Data acquisition was performed in full-scan mode (50–1000 Da) with a scan time of 0.5 s and a collision energy of 6 eV; argon was used as the collision gas (optimized pressure of 5×10^{-3} mbar). Analyses were performed in positive mode (ESI^{+}), therefore protonated molecules $[M+H]^{+}$ were collected in each MS spectrum. HPLC–UV purity was determined for every prepared compound. The percentage result was calculated as the representation of the molecular peak area compared to the sum of the remaining peak areas in the entire HPLC–UV spectrum.

2.1.3. HRMS conditions

Samples were prepared as described above (*HPLC–MS conditions*). Samples (5 µl) were injected onto a reversed-phase column (Symmetry C18, 5 µm, 150 mm × 2.1 mm; Waters, Milford, MA, USA) incubated at 40°C. Solvent A was 15 mM ammonium formate adjusted to pH 4.0. Solvent B was methanol. The following linear gradient was used at a flow rate of 250 µl min^{-1}: 0 min, 10% B; 0–15 min, 90% B. The effluent was introduced to a DAD detector (scanning range 210–400 nm with 1.2 nm resolution) and then to an electrospray source (source temperature 150°C, desolvation

temperature 550°C, capillary voltage 1 kV, cone voltage 25 V). Nitrogen was used as the cone gas (50 l h^{-1}) and the desolvation gas (1000 l h^{-1}). Data acquisition was performed in full-scan mode (50–1000 Da) with a scan time of 0.5 s and collision energy of 4 eV; argon was used as the collision gas (optimized pressure of 5×10^{-3} mbar). Analyses were performed in positive mode (ESI^+), therefore protonated molecules $[M+H]^+$ were collected in each MS spectrum. For the exact mass determination experiments, the external calibration was performed using lock spray technology and a mixture of leucine/encephalin (50 pg μl^{-1}) in an acetonitrile and water (1 : 1) solution with 0.1% formic acid as a reference. Accurate masses were calculated and used to determine the elemental composition of the analytes with a fidelity better than 1.0 ppm.

2.1.4. Calculating isotopologue abundance

The isotopologue composition of each prepared compound was calculated as described below. HRMS analysis was performed to obtain the most accurate spectra. Subsequently, every isotopologue was identified and its presence in proportion to the whole mixture was calculated. For instance, the MS spectra in figure 1 demonstrate isotopologue enumeration for 6-amino-9*H*-[$^{15}N_5$]-purine. However, an isotopologue abundance calculation method was subsequently applied to each synthesized compound.

2.2. Chemicals

[^{15}N]-formamide (99.1% ^{15}N enrichment based on starting materials) was obtained from Cambridge Isotope Laboratories (Andover, USA). *N*,*N*-diisopropylethylamine (DIPEA), *tert*-butyl methyl ether (M*T*BE), 3-methoxybenzylamine, benzylamine, DMSO-d6 and Dowex 50 W were obtained from Sigma-Aldrich. Phosphorus oxychloride ($POCl_3$) was obtained from Merck Millipore. Lachner supplied *n*-propanol and acetic acid (CH_3COOH). Penta supplied ammonium hydroxide solution (NH_4OH), sodium nitrite ($NaNO_2$), triethylamine, methanol and chloroform. Olchemim Ltd (Olomouc, Czech Republic) supplied 3-methylbut-2-en-1-amine hydrochloride, 4-amino-2-methylbut-2-en-1-ol hemitrate salt, 2-hydroxy-, 3-hydroxy- and 4-hydroxybenzylamines. Milli-Q water was used throughout. The other solvents and chemicals used were all of standard p.a. quality.

2.3. Synthesis

2.3.1. 6-amino-9*H*-[$^{15}N_5$]-purine (adenine) (**1**)

[$^{15}N_5$]-adenine was prepared by cyclization of [^{15}N]-formamide in the presence of $POCl_3$, as previously described [35]. A reaction mixture containing [^{15}N]-formamide (6.0 ml; 0.15 M) and $POCl_3$ (28.2 ml; 0.30 M) was placed into a stainless-steel reactor with a polytetrafluoroethylene (PTFE) tube insert and stirred for 17 h at 130°C under argon. After cooling, the mixture was transferred to a flask containing Dowex 50 W (H^+ form, 150 g) and water (200 ml). The contents were extensively washed with water and the product was eluted using NH_4OH (5 M) after 2 days of stirring, as described [22]. Next, the solution was evaporated to constant weight. Owing to the already-described occurrence of the stereoisomer 1H-imidazo-[4,5-b]pyrazine-5-amine [36], the reaction mixture was finally purified by flash chromatography on a silica gel column using chloroform/methanol/ammonia (6 : 1 : 0.05) as the mobile phase. Collected fractions were evaporated again to give the final product. Yield: 1.5 g white crystal (32%). TLC (chloroform/methanol/ammonia 6 : 1 : 0.05, v/v/v): one single spot, free of 1H-imidazo-[4,5-b]pyrazine-5-amine. Melting point 372–375°C. HRMS (ESI^+): *m*/*z* 141.0475 $[M+H]^+$ (Calcd for $[C_5H_5^{15}N_5+H]^+$ 141.0475). MS (ESI^+): *m*/*z* 140.89 $[M+H]^+$. HPLC–UV purity: 98+%. $^{15}N_5$ isotopologue abundance: 95.0%. ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 6.98 (br. s., 1 H, HN-6) 7.15 (br. s., 1 H, HN-6) 8.00–8.11 (m, 2 H, H-2, H-8) 12.81 (br. s., 1 H, HN-9). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 119.1 (C-5), 139.5 (C-8), 150.6 (C-4), 152.8 (C-2), 156.1 (C-6). ^{15}N NMR (51 MHz, DMSO-d_6) δ ppm 75.3 (d, $J = 4.6$ Hz, 1 N, N-6) 153.3 (s, 1 N, N-9) 223.8 (s, 1 N, N-3) 230.3 (d, $J = 5.2$ Hz, 1 N, N-1) 236.4 (s, 1 N, N-7).

2.3.2. 1,7-dihydro-6*H*-[$^{15}N_4$]-purine-6-one (hypoxanthine) (**2**)

[$^{15}N_4$]-hypoxanthine was prepared by [$^{15}N_5$]-adenine deamination in a weak acidic medium containing nitric salts, as previously described [37]. Sodium nitrite (4.5 g; 0.06 M) was added to the suspension of [$^{15}N_5$]-adenine (1.5 g; 0.01 M) and acetic acid (30%; 41 ml; 0.2 M), and the mixture was heated to 40°C

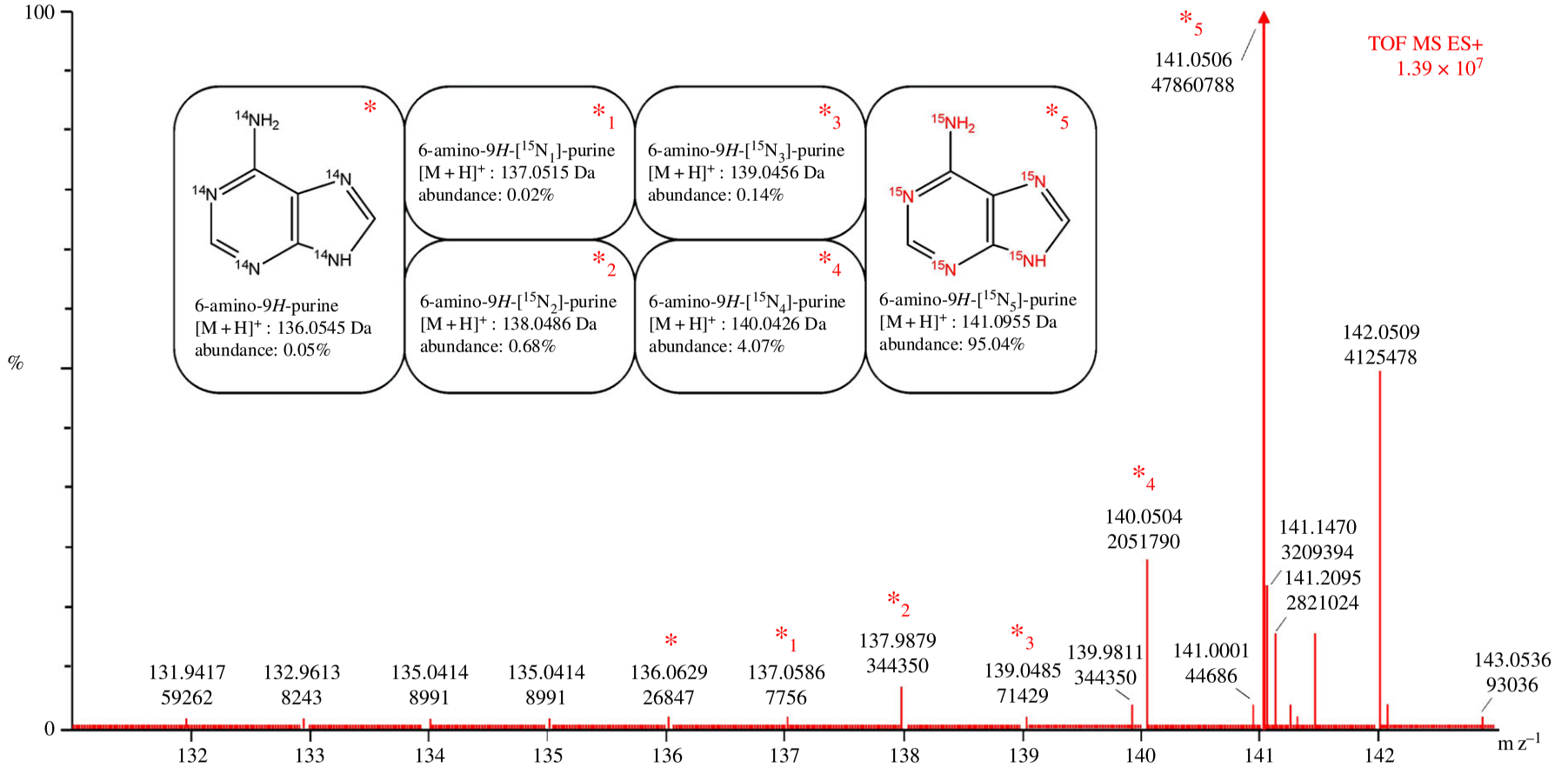


Figure 1. HRMS spectra of 6-amino-9H-[$^{15}N_5$]-purine and its respective isotopologues. Theoretical monoisotopic weights of protonated form for identifying molecular ion peak and calculated isotopologue abundance are mentioned.

and stirred for 1 h. Next, the reaction mixture was left to crystallize at 4°C. After crystallization, the precipitate was filtered off, washed with water (3 × 5 ml) and dried at 60°C to constant weight. Yield: 1.1 g white crystal (71%). TLC (chloroform/methanol/ammonia 6:1:0.05, v/v/v): one single spot, free of starting compound. HRMS (ESI^+): *m/z* 141.0345 $[M+H]^+$ (Calcd for $[C_5H_4^{15}N_4O+H]^+$ 141.0345). MS (ESI^+): *m/z* 141.06 $[M+H]^+$. HPLC–UV purity: 97+%. ^1H NMR (500 MHz, DMSO-d6) δ ppm 7.81–8.00 (m, 1 H, H-2) 8.06 (br. s., 1 H, H-8) 12.09 (br. s., 1 H) 12.27 (br. s., 1 H). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 115.8 (C-5), 139.1 (C-8), 142.4 (C-2), 144.8 (C-6), 157.5 (C-4).

2.3.3. 6-chloro-9*H*-[$^{15}N_4$]-purine (6-chloropurine) (**3**)

[$^{15}N_4$]-6-chloropurine preparation was based on chlorination of [$^{15}N_4$]-hypoxanthine, as previously described [38]. DIPEA (3.1 ml; 0.02 M) was slowly added to a mixture of [$^{15}N_4$]-hypoxanthine (1.1 g; 7.8 mM) and $POCl_3$ (44 ml; 0.47 M), and the reaction was stirred under reflux for 4 h at 130°C. $POCl_3$ was then removed by distillation under reduced pressure. The $POCl_3$-free solution was subsequently transferred into a flask containing 15 ml of MTBE and an equal volume of cold water while stirring for 30 min. The water phase was removed and then subjected to extraction using MTBE again a total of 10 times (10 × 15 ml). Organic fractions collected were evaporated to constant weight. Yield: 1.2 g slightly yellow crystal (95%). TLC (chloroform/methanol/ammonia 6 : 1 : 0.05, v/v/v): one single spot, free of starting compound. Melting point 177°C. HRMS (ESI^+): *m/z* 159.0006 $[M+H]^+$ (Calcd for $[C_5H_3^{15}N_4Cl+H]^+$ 159.0006). MS (ESI^+): *m/z* 158.81 $[M+H]^+$. HPLC–UV purity: 97+ %. ^1H NMR (500 MHz, DMSO-d_6) δ ppm 8.63 (s, 1 H, H-8) 8.68 (s, 1 H, H-2) 12.23 (br. s., 1H, HN-9). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 129.7 (C-5), 146.7 (C-8), 148.2 (C-6), 152.0 (C-2), 154.5 (C-4). ^{15}N NMR (51 MHz, DMSO-d_6) δ ppm 173.15 (s, 1 N, N-9) 224.4 (s, 1 N, N-7) 251.90 (s, 1 N, N-3) 268.78 (s, 1 N, N-1).

2.4. Synthesis of [$^{15}N_4$] aromatic cytokinins

General procedures for preparing non-labelled monohydroxylated 6-benzylaminopurines (**4**, **5**, **6**) have previously been described [18]. Preparation methods for 6-(3-methoxybenzylamino)-9*H*-purine (**7**) and 6-benzylamino-9*H*-purine (**8**) have also been previously published [6,39]. In general, preparation of corresponding substituted ARCKs was based on [$^{15}N_4$]-6-chloropurine (100 mg) reaction with the appropriate amine and triethylamine (molar ratio 1 : 1 : 2) in cold (0°C) *n*-propanol at 100°C for 5 h in an inert atmosphere (Ar). After cooling, the reaction mixture was left to crystallize at room temperature for 24 h. Then, the precipitate was filtered out, washed with *n*-propanol (3 × 5 ml) and water (3 × 5 ml) and dried at 60°C to constant weight. Yields and analytical data (TLC; HRMS; HPLC–MS; ^1H, ^{13}C NMR) are mentioned in the relevant section below.

2.4.1. 6-(4-hydroxybenzylamino)-9*H*-[$^{15}N_4$]-purine (para-topolin, pT) (**4**)

Yield: 124.1 mg white crystal (80%). TLC (ethyl acetate/methanol/ammonia 34 : 4 : 2, v/v/v): one single spot, free of starting compound. HRMS (ESI^+): *m/z* 246.0924 $[M+H]^+$ (Calcd for $[C_{12}H_{11}N^{15}N_4O+H]^+$ 246.0923). MS (ESI^+): *m/z* 246.11 $[M+H]^+$. HPLC–UV purity: 98+ %. ^1H NMR (500 MHz, DMSO-d6) δ ppm 4.52 (br. s., 2 H) 6.62 (m, *J* = 8.41 Hz, 2 H) 7.11 (m, *J* = 8.41 Hz, 2 H) 7.91–8.08 (m, 2 H) 8.13 (t, *J* = 15.21 Hz, 1 H) 9.22 (br. s., 1 H) 12.62 (br. s., 1 H). ^{13}C NMR (126 MHz, DMSO-d6) δ ppm 42.95, 115.43, 119.13, 129.15, 130.82, 139.37, 149.85, 152.83, 154.60, 156.63.

2.4.2. 6-(2-hydroxybenzylamino)-9*H*-[$^{15}N_4$]-purine (ortho-topolin, oT) (**5**)

Yield: 120.9 mg white crystal (78%). TLC (ethyl acetate/methanol/ammonia 34 : 4 : 2, v/v/v): one single spot, free of starting compound. HRMS (ESI^+): *m/z* 246.0923 $[M+H]^+$ (Calcd for $[C_{12}H_{11}N^{15}N_4O+H]^+$ 246.0923). MS (ESI^+): *m/z* 245.99 $[M+H]^+$. HPLC–UV purity: 99+ %. ^1H NMR (500 MHz, DMSO-d6) δ ppm 4.53 (br. s., 2 H) 6.67 (t, J = 7.37 Hz, 1 H) 6.75 (d, *J* = 7.95 Hz, 1 H) 7.01 (t, *J* = 7.49 Hz, 1 H) 7.09 (d, *J* = 7.34 Hz, 1 H) 7.99–8.12 (m, 2 H) 8.12–8.20 (m, 1 H) 10.09 (br. s., 1 H) 12.78 (br. s., 1 H). ^{13}C NMR (126 MHz, DMSO-d6) δ ppm 116.07, 119.44, 126.27, 128.48, 129.22, 139.69, 149.90, 152.52, 154.44, 155.57. ^{15}N NMR (51 MHz, DMSO-d6) δ ppm 153.8, 220.6, 223.4, 235.7.

2.4.3. 6-(3-hydroxybenzylamino)-9*H*-[$^{15}N_4$]-purine (meta-topolin, mT) (**6**)

Yield: 85.4 mg white crystal (55%). TLC (ethyl acetate/methanol/ammonia 34 : 4 : 2, v/v/v): one single spot, free of starting compound. HRMS (ESI^+): *m/z* 246.0925 $[M+H]^+$ (Calcd for $[C_{12}H_{11}N^{15}N_4O+H]^+$

246.0923). MS (ESI$^+$): *m/z* 246.09 [M+H]$^+$. HPLC–UV purity: 98+ %. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 3.62 (br. s., 1 H) 4.53 (br. s., 1 H) 6.49 (br. s., 1 H) 6.65 (br. s., 2 H) 6.97 (d, $J = 7.26$ Hz, 1 H) 8.06 (br. s., 2 H) 9.19 (br. s., 1 H) 12.84 (br. s., 1 H). ^{13}C NMR (126 MHz, DMSO-d6) δ ppm 43.15, 113.98, 114.37, 118.23, 129.64, 139.28, 139.35, 142.25, 150.06, 152.86, 154.73, 157.78.

2.4.4. 6-(3-methoxybenzylamino)-9*H*-[$^{15}N_4$]-purine (meta-methoxytopolin, memT) (**7**)

Yield: 139.4 mg white crystal (85%). TLC (ethyl acetate/methanol/ammonia 34 : 4 : 2, v/v/v): one single spot, free of starting compound. HRMS (ESI$^+$): *m/z* 260.1080 [M+H]$^+$ (Calcd for [$C_{13}H_{13}N^{15}N_4O$+H]$^+$ 260.1080). MS (ESI$^+$): *m/z* 260.07 [M+H]$^+$. HPLC–UV purity: 99+ %. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 3.65 (s, 3 H, O-CH_3) 4.62 (br. s., 2 H, N-CH_2) 6.72 (d, $J = 7.1$ Hz, 1 H, C_{Ar}) 6.84–6.89 (m, 2 H, C_{Ar}) 7.15 (t, $J = 7.8$ Hz, 1 H, C_{Ar}) 8.02–8.09 (m, 1 H, H-8) 8.13 (d, $J = 15.9$ Hz, 1 H, H-2) 12.91–12.99 (br. m, 1H, HN-9). ^{13}C NMR (126 MHz, DMSO-d6) δ ppm 43.27, 55.41, 112.27, 113.47, 119.84, 129.75, 139.32, 139.40, 142.41, 149.98, 152.83, 154.74, 159.71.

2.4.5. 6-benzylamino-9*H*-[$^{15}N_4$]-purine (BAP) (**8**)

Yield: 59.5 mg white crystal (41%). TLC (ethyl acetate/methanol/ammonia 34 : 4 : 2, v/v/v): one single spot, free of starting compound. HRMS (ESI$^+$): *m/z* 230.0984 [M+H]$^+$ (Calcd for [$C_{12}H_{11}N^{15}N_4O$+H]$^+$ 230.0974). MS (ESI$^+$): *m/z* 230.00 [M+H]$^+$. HPLC–UV purity: 99+ %. ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 4.65 (br. s., 2 H, N-CH_2) 7.11–7.19 (m, 1 H, *p*-H_{Ar}) 7.23 (t, $J = 7.5$ Hz, 2 H, *o*-H_{Ar}) 7.29 (d, $J = 7.34$ Hz, 2 H, *m*-H_{Ar}) 8.01–8.09 (m, 1 H, H-2) 8.09–8.26 (m, 2 H, H-8, HN-6) 12.81–12.99 (br. m, 1 H, HN-9). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 43.3 (NH-CH_2), 127.0 (C_{Ar}), 127.6 (C_{Ar}), 128.7 (C_{Ar}), 139.3 (C-8), 140.7 (C-4), 150.0 (C_{Ar}), 152.8 (C-2), 154.7 (C-6).

2.5. Synthesis of [$^{15}N_4$] isoprenoid cytokinins

The preparation of 6-[(3-methylbut-2-en-1-yl)]-9*H*-purine-6-amine (**9**) has previously been described [7] and its isotopically labelled analogue was prepared in a similar manner. [$^{15}N_4$]-6-chloropurine (50 mg; 0.3 mM) was dissolved in *n*-propanol (830 µl; 11 mM), and (3-methylbut-2-en-1-yl)amine hydrochloride (39 mg; 0.4 mM) was added in the presence of triethylamine (181 µl; 1.3 mM). The reaction was performed at 100°C for 5 h in an inert atmosphere (Ar). After cooling to room temperature, crystallization was immediately observed in the reaction mixture. The resulting crystal was filtered out, washed with *n*-propanol (3 × 2 ml) and water (3 × 2 ml) and dried at 60°C to constant weight. Melting point 201–204°C.

The syntheses of 6-[(*E*)-4-hydroxy-3-methylbut-2-en-1-yl]-9*H*-[$^{15}N_4$]-purine-6-amine (**10**) and 6-[(*Z*)-4-hydroxy-3-methylbut-2-en-1-yl]-9*H*-[$^{15}N_4$]-purine-6-amine (**11**) were based on the original protocols [15] with slight modifications. These syntheses were generally based on the reaction of [$^{15}N_4$]-6-chloropurine (50 mg) with the appropriate amine in the presence of DIPEA (molar ratio 1 : 2 : 4) and excess methanol at 85°C for 48 h in an inert atmosphere (Ar) in a pressure tube. The reaction solvents were then evaporated and replaced with water. The product was then crystallized from water at reduced temperature for 48 h. Yields and analytical data (TLC; HRMS; HPLC–MS; ^{1}H, ^{13}C NMR) are mentioned in the relevant section below.

2.5.1. 6-[(E)-4-hydroxy-3-methylbut-2-en-1-yl]-9*H*-[$^{15}N_4$]-purine-6-amine (*trans*-zeatin, tZ) (**10**)

Yield: 28.2 mg white crystal (40%). TLC (chloroform/methanol 86 : 14, v/v): one single spot, free of starting compound. Melting point 198–200°C. HRMS (ESI+): *m/z* 224.1082 [M+H]$^+$ (Calcd for [$C_{10}H_{13}N^{15}N_4O$+H] + 224.1080). MS (ESI+): *m/z* 224.10 [M+H]$^+$. HPLC–UV purity: 97+ %. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 1.61 (s, 3 H, CH_3) 3.73 (br. s., 2 H, CH_2-O) 4.05 (br. s., 2 H, NH-C$\underline{H_2}$) 4.70 (br. s., 1 H, -OH) 5.47 (br. s., 1 H, CH=) 7.66 (br. s., 1 H, $\underline{H}$N-CH_2) 7.96–8.06 (m, 1 H, H-8) 8.11 (t, $J = 14.90$ Hz, 1 H, H-2) 12.93 (br. s., 1 H, HN-9). ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 14.1 (CH_3), 37.7 (N-CH_2), 66.3 (CH_2-O), 119.2 (C-5), 121.4 (CH=), 137.6 (C=), 139.0 (C-8), 149.9 (C-4), 152.8 (C-2), 154.6 (C-6).

2.5.2. 6-[(Z)-4-hydroxy-3-methylbut-2-en-1-yl]-9*H*-[$^{15}N_4$]-purine-6-amine (*cis*-zeatin, cZ) (**11**)

Yield: 29.0 mg white crystal (41%). TLC (chloroform/methanol 86 : 14, v/v): one single spot, free of starting compound. HRMS (ESI+): *m/z* 224.1080 [M+H]$^+$ (Calcd for [$C_{10}H_{13}N^{15}N_4O$+H] + 224.1080).

MS (ESI+): *m*/*z* 224.00 $[M+H]^+$. HPLC–UV purity: 96+ %. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 1.64 (s, 3 H) 3.98 (s, 2 H) 4.06 (br. s., 2 H) 4.72 (br. s., 1 H) 5.29 (br. s., 1 H) 7.59 (br. s., 1 H) 7.98–8.21 (m, 2 H) 12.77–12.94 (br. s., 1 H). ^{13}C NMR (126 MHz, DMSO-d6) δ ppm 21.64, 37.32, 55.31, 60.16, 123.80, 137.98, 139.09, 149.75, 152.69, 154.41.

3. Results and discussion

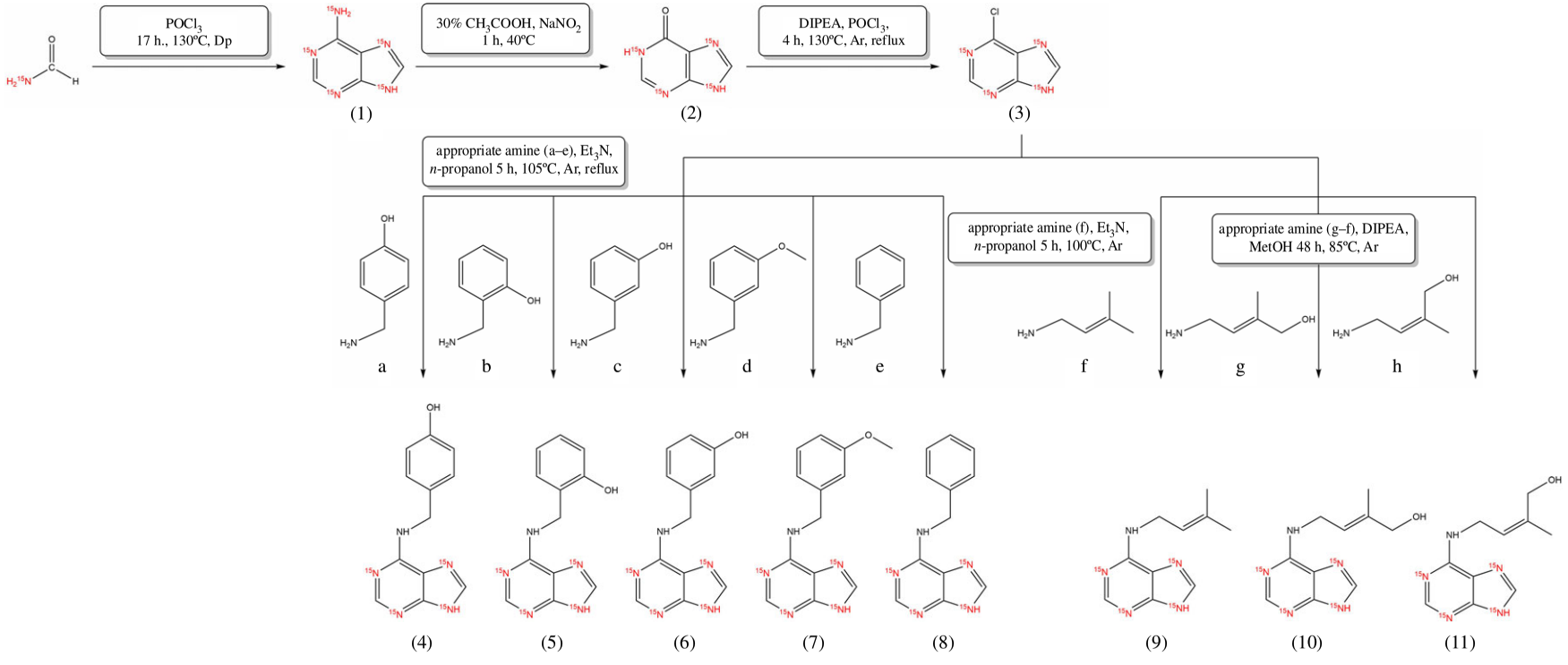
Eleven [$^{15}N_4$]-core-labelled purine derivatives were prepared in this work, from which six have not been prepared previously. Excluding the starting compounds and intermediates (**1**, **2**, **3**), five aromatic (**4**, **5**, **6**, **7**, **8**) and three (**9**, **10**, **11**) isoprenoid cytokinins were synthesized. The identity and purity of each prepared compound were verified by TLC, HPLC–UV–DAD/HPLC–MS, HRMS, and ^{1}H, ^{13}C and ^{15}N NMR. Melting point data, measured for some of the prepared compounds, were in very good agreement with previously published data for their unlabelled counterparts, although a little bit lower [40,41], probably due to isotopic effect. ^{15}N NMR analyses were performed only for highly important intermediates (**1**, **3**) and one aromatic cytokinin, ortho-topolin **(5)**, as an example of the end-product. The ^{15}N NMR shifts obtained for **1** and **3** are in good agreement with literature data [34,42]. When $CH_3^{15}NO_2$ was used in literature for shift calibration, then this shift was corrected by 380.5 ppm for comparison with the ^{15}N shift obtained with calibration on liquid $^{15}NH_3$. The abundance of major isotopologues is mentioned in details also only for parent molecule (**1**) to confirm the presumed transfer of the ^{15}N nuclide from commercially available [^{15}N]-formamide. Isotopologic profiles of the remaining prepared compounds confirmed theoretical expectations. The abundance of the non-labelled forms of each prepared compound was less than 0.2% (data not shown). Synthesis of some compounds in their non-labelled form had previously been described [6,7,15,17,35,37,38]. However, most synthesis procedures were optimized significantly to synthesize labelled compounds on minimalistic scales. Thus, the number of purifications and other post-reaction steps were reduced in effort to produce reasonable yields. Scheme 1 summarizes synthesis work flows and detailed reaction conditions. Table 1 presents the yields obtained, HPLC–UV purities and the results of HPLC–MS and HRMS analyses.

The preparation of pure, fully labelled parent molecule **1** was critical for later synthesis. The reaction between formamide and $POCl_3$ in a 1 : 2 molar ratio and under defined conditions should give product (**1**) [35]. However, after HPLC–UV, mass spectrometry and ^{1}H NMR analyses (data not shown), a contaminant, 1*H*-imidazo-[4,5-b]-pyrazine-5-amine (**1b**) was discovered in a 1 : 1 ratio with adenine (**1**). Authors [35] did not observe the formation of this contaminant. A study published 10 years later using the same reaction conditions confirmed the production of **1b** but also presented a methodology for its elimination based on stereospecific transformation of **1** to its picrate salts. Using this improved method, 93% of **1** contained in the reaction mixture should be isolated [36]. Unfortunately, after several repetitions, we were unable to obtain similar yield. Based on the results of HPLC–MS analyses (data not shown), **1** was not fully isolated. However, whether the method by [13] applies at the milligram scale used for our approach is questionable.

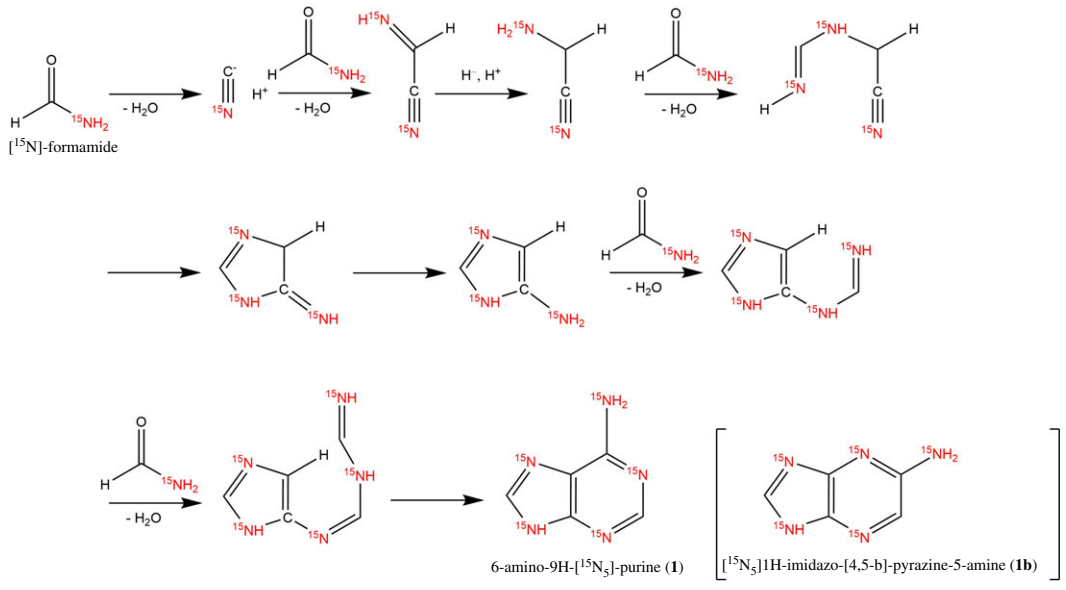
Based on this consideration, we decided to focus on developing a more effective, single-stage separation method while maintaining the starting conditions described by [35]. Reactions were performed in a stainless-steel reactor with a PTFE tube insert, which led to increased reaction stability and homogeneity. Subsequently, $POCl_3$ was removed by distillation. The reaction mixture was subjected to ion exchange chromatography performed by Dowex 50 W (H+ form) for 48 h due to the elimination of unreacted intermediates formed during supposed multistep cyclization of 1 (scheme 2) [43]. Elimination of these unreacted intermediates is crucial for the flawless course of later steps. Next, **1** and **1b** were eluted using 5 M NH_4OH as described elsewhere [22]. Finally, the reaction mixture containing **1** (65.8%) and **1b** (31.6%) was subjected to column chromatography using chloroform/methanol/ammonia (6 : 1 : 0.05) as the mobile phase. The final product (**1**) was isolated with a total yield of 32% and HPLC–UV purity of 98%.

Fractions containing **1b** were collected and subjected to HPLC–UV–DAD/HPLC–MS analysis to confirm chemical identity. Figure 2 shows HPLC chromatograms of **1** and **1b** isolation. Considering some of the previously published procedures for preparing **1** from simple precursors, relatively low yields are typical for this approach, due to multistep cyclization with several intermediates [22,35,36]. However, for further preparations of isotopically labelled CKs, high chemical purity and isotopic enrichment are especially crucial.

Since isotopically labelled CKs were the target products of our synthesis approach, **3** played a crucial role as the acceptor of aromatic or isoprenoid side chains during nucleophilic substitution at the



Scheme 1. Reaction scheme of the preparation of [$^{15}N_4$]-labelled purine derivatives. Detailed reaction conditions are mentioned in the brackets. (*a*) 4-hydroxybenzylamine; (*b*) 2-hydroxybenzylamine; (*c*) 3-hydroxybenzylamine; (*d*) 3-methoxybenzylamine; (*e*) benzylamine; (*f*) 3-methylbut-2-en-1-amine; (*g*) (E)-4-amino-2-methylbut-2-en-1-ol; (*h*) (Z)-4-amino-2-methyl-2-buten-1-ol.



Scheme 2. Supposed multistep 6-amino-9H-[$^{15}N_5$]-purine (**1**) cyclization from [^{15}N]-formamide. [$^{15}N_5$]1H-imidazo-(4,5-b)-pyrazine-5-amine (**1b**) shown in brackets because its cyclization pathway is supposedly similar to that of **1** but is not further investigated herein [43].

Table 1. Yields and results of physico-chemical analyses performed for each of the prepared compounds.

				high resolution mass spectrometry			
compound	yield (%)	HPLC purity (%)	MS $[M+H]^+$	measured mass $[M+H]^+$	calculated mass $[M+H]^+$	molecular formula	fidelity (ppm)
1	32	98[a]	140.89	141.0475	141.0475	$C_5H_5^{15}N_5$	0.0
2	71	97[b]	141.06	141.0345	141.0345	$C_5H_4^{15}N_4O$	0.0
3	95	97[c]	158.81	159.0006	159.0006	$C_5H_3^{15}N_4Cl$	0.0
4	80	98[b]	246.11	246.0924	246.0923	$C_{12}H_{11}N^{15}N_4O$	0.4
5	78	99[b]	245.99	246.0923	246.0923	$C_{12}H_{11}N^{15}N_4O$	0.0
6	55	98[b]	246.09	246.0925	246.0923	$C_{12}H_{11}N^{15}N_4O$	0.8
7	85	99[b]	260.07	260.1080	260.1080	$C_{13}H_{13}N^{15}N_4O$	0.7
8	41	99[b]	230.00	230.0984	230.0974	$C_{12}H_{11}N^{15}N_4O$	0.4
9	40	99[b]	207.90	208.1132	208.1131	$C_{10}H_{13}N^{15}N_4$	0.5
10	40	97[d]	224.10	224.1082	224.1080	$C_{10}H_{13}N^{15}N_4O$	0.9
11	41	96[d]	224.00	224.1080	224.1080	$C_{10}H_{13}N^{15}N_4O$	0.0

[a]Purified by column chromatography.
[b]Crystallization from reaction mixture.
[c]Purified by *tert*-butyl methyl ether: H_2O extraction.
[d]Purified by crystallization from H_2O.

C^6-position. For this reason, we made significant efforts to obtain **3** in maximal yield and purity, and simultaneously tried to reduce the number of post-reaction steps. The preparation of **3** is generally based on hypoxanthine chlorination in the presence of the appropriate base and chlorine donor [38]. While phosphorus oxychloride ($POCl_3$) is typically the chlorine donor, choosing a suitable base is a matter of optimization. Approaches using *N*,*N*-dimethylaniline (DMA) [38,44], as well as those in which the reaction is conducted without the base, are known [45].

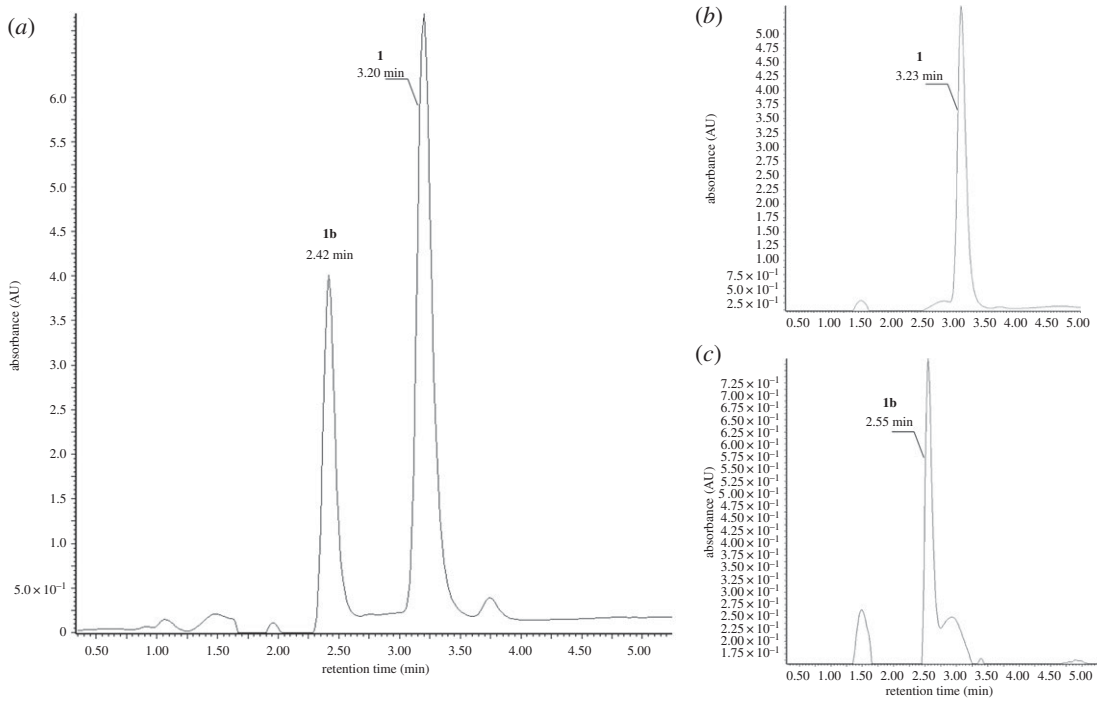


Figure 2. HPLC–UV chromatograms of a non-purified mixture (*a*) of [$^{15}N_5$]-adenine (1) and [$^{15}N_5$]-1H-imidazo-[4,5-b]-pyrazine-5-amine (1b). After application of column chromatography separation using chloroform/methanol/ammonia (6 : 1 : 0.05) as the mobile phase, fully separated 1 and 1b were observed as evident from *b* and *c*, respectively.

Owing to the apparent simplicity and relatively good yields (53%) of the base-less approach, we tried it several times. However, our attempts at this approach did not lead to the desired product. Therefore, we increased the reaction temperature from 65°C to 100°C. This temperature increase produced **3** with sufficient HPLC–UV purity (97%) but less than 10% yield.

Subsequent attempts to synthesize **3** were based on the approach in [22,38] but with some modifications. DMA was at first replaced with triethylamine (TEA), the temperature was increased from room temperature to 105°C, the reaction time was reduced from 24 h to 4 h, and finally, the reaction was followed by discontinuous extraction in an ethylacetate : H_2O system (1 : 1). These attempts produced **3** with a 14% yield, and HPLC–UV purity of 85% with no further purifications. Reaction conditions were further modified to increase the yield, so a 48 h long continuous extraction using diethyl ether was introduced. As expected, the yield increased up to 26%, while HPLC–UV purity remained below 90%.

Considering the results of previous optimization attempts, the duration of the whole method (72 h), and instrumental difficulties (continuous extraction, several pH adjustments, etc.), we shifted our attention to developing more efficient, more reliable and simpler methods. Our main goals were to select a functional base and to avoid time-consuming product extraction. The chlorine donor, $POCl_3$, was preserved, and reaction conditions, i.e. a temperature of 130°C and a duration of 4 h, were established. We tried 2,6-dimethylpyridine as a base at first, but with no success. Owing to its wide application in organic chemistry, *N*,*N*-diisopropylethylamine (DIPEA, Hünigs base) was finally used, instead of TEA. Selection of hypoxanthine : $POCl_3$: DIPEA (1 : 6 : 2) at defined reaction conditions, together with subsequent discontinuous product extraction by *tert*-butyl methyl ether (*MTBE*) : H_2O system (1 : 1) led to the desired production of **3** with 95% yield and HPLC–UV purity of 97%. This simple and very efficient method for preparing **3** with a reaction time shorter than 8 h is presented herein. Table 2 summarizes the results of optimization, including the partial results according to the reaction conditions.

The final step in the synthesis of aromatic and isoprenoid CKs was conducted as previously published in the literature without any major changes. The preparation of **8** was performed either with a non-substituted ring [39] or accompanied by various monohydroxy- (**4**, **5**, **6**) or monomethoxy- (**7**) substituents at the phenyl ring [6,39]. Isoprenoid CKs were prepared using previously published procedures (**9**) or with slight modifications in which TEA was replaced with DIPEA and the reaction time was extended up to 48 h (**10**, **11**) [7,15]. Since any significant modifications to the preparation methods were made to the last reaction step (C^6-conjugation), the yields and purities of all newly

Table 2. Results of the optimization process of 1,7-dihydro-6H-($^{15}N_4$)-purine-6-one (hypoxanthine, 1) chlorination to 6-chloro-9H-($^{15}N_4$)-purine (6-chloropurine, 2).

reactants[a]	reaction conditions		additional purification step	yield (%)	HPLC purity (%)
	temperature (°C)	time (h)			
acetonitrile, ethylbenzene	65	6	*a*	n.d.	n.d.
acetonitrile, ethylbenzene	65–80	6	*a*	n.d.	n.d.
acetonitrile, ethylbenzene	100	6	*a*	<10	97
triethylamine	105	4	*b*	14	85
triethylamine	105	4	*c*	25	<90
2,6-dimethylpyridine	130	4	*a*	n.d.	n.d.
N,*N*-diisopropylethylamine	130	4	*d*	95	97

[a]Including hypoxanthine and $POCl_3$ in all cases.

a, none; *b*, discontinuous extraction by ethylacetate : H_2O (1 : 1); *c*, continuous extraction (48 h) by diethyl ether; *d*, discontinuous extraction by *tert*-butyl methyl ether : H_2O (1 : 1).

synthesized compounds were comparable to those mentioned in the literature [7,15]. The consequences of isotopic labelling with ^2H, ^{13}C and especially ^{15}N, used in this work, will be further discussed below.

Generally, CKs labelled with deuterium or ^{13}C nuclide are most frequently prepared [16,20,21,23–25]. For ^{15}N labelling, to the best of our knowledge, for aromatic cytokinins only the preparation of [$^{15}N_1$]-BAP has been described in the literature [17,18]. The only [$^{15}N_4$]-labelled cytokinin prepared previously was *trans*-zeatin and its riboside, synthesized by Horgan & Scott [22]. Their basic strategy was similar to ours; however, with very low yield and unreported isotopic purity of the final products. The prevalence of approaches that use deuterium or ^{13}C nuclide to prepare isotopically labelled CKs is reasonable. Deuterium labelling is relatively inexpensive and can be accomplished using deuterated catalytic reduction systems [23,25] or catalysed hydrogen-deuterium exchange [16]. The ^{13}C nuclide could easily be integrated by using ^{13}C reagents [20,23,24].

However, use of ^2H or ^{13}C nuclides has its disadvantages. First, most of the preparation methods mentioned are based on incorporation of one [20,23,24] or approximately two [20,23] isotopes. Truthfully, there are some preparation methods for CKs labelled with multiple isotopes. [$^2H_5$]-*t*Z can be obtained by using [$^2H_6$]-acetone as a starting compound for the preparation of (*E*)-4-amino-1,1-[$^2H_2$]-2-[$^2H_3$]-methylbut-2-en-1-ol, which is subsequently reacted with 6-chloropurine to get the desired ISCK. Additionally, [$^2H_4$]-BAP can be obtained by a hydrogen–deuterium exchange reaction catalysed by palladium on a carbon-ethylene diamine complex [Pd/C(en)]. In this method, the hydrogen–deuterium exchange occurs most frequently at C2, C8 (94%) and side chain carbon positions (97%) [16].

It is noteworthy that the preparation methods described are based on integration of approximately two nuclides into the purine core. If there are more isotopes in the structure, they are typically located on the side chain. Side chain locations of isotopes could be problematic for further biological application because, while a purine core is relatively stable during metabolism, side chains are often being transformed. Therefore, for biological application, the weights of compounds prepared with side chain isotopes are displaced by an approximate two-unit weight shift. This shift casts doubt on the reliability of these compounds for use as internal standards in MS [12].

Moreover, use of ^2H nuclides can introduce further complications due to their different physico-chemical properties compared to naturally occurring nuclides. Specifically, the literature explains that some deuterated forms of drugs demonstrate different transport processes, increased resistance to metabolic change, or even changes to the entire pathway of its metabolism [33]. Moreover, use of

deuterated internal standards can cause unstable retention times associated with the number of deuterium atoms used. The so-called deuterium isotope effect can lead to the worst accuracy and precision for a quantification method [46].

The method presented herein provides full purine-core-labelled CKs with a stable ^{15}N nuclide that is free of side effects and that produces target compounds with high yield efficiency and high chemical purities. High abundance of the most enriched isotopologue, with the non-labelled form present at under 0.05% abundance, is further guaranteed by ^{15}N nuclide stability as well as full purine-core cyclization at the beginning of the whole approach using simple [^{15}N]-formamide as the starting compound. Thus, space for incorporation of natural nitrogen isotopes is minimized.

Since every prepared adenine derivative contains at least four ^{15}N atoms at the stable purine-core positions, they are fully useful for MS applications [47]. Moreover, this preparation method is applicable for the preparation of any other desired [^{15}N]-labelled C6-substituted purine derivatives. Although the applications of some compounds discussed or presented in this paper have already been published [47], a preparation method for these compounds is presented here for the very first time.

4. Conclusion

In summary, 11 [$^{15}N_4$]-core-labelled purine derivatives were synthesized, including five ARCKs (**4**, **5**, **6**, **7** and **8**) and three ISCKs (**9**, **10** and **11**). Effective modifications of previously published procedures led to enhanced selectivity of product preparation for **1** and more effective overall synthesis of **3**. According to the results of the analyses performed, the identity of all 11 compounds was confirmed and their purity proved sufficient for further applications. Moreover, the approaches presented are applicable for synthesizing any other desired [^{15}N]-labelled C6-substituted purine derivatives.

Data accessibility. This article has no additional data: all the data and other materials required to allow a reader to perform a full replication are available in the main body of the manuscript.

Authors' contributions. M.Z., L.H., K.D. and M.S. conceived the research idea and designed the experiments. J.B., M.Z., L.H. and L.P. participated on synthesis; T.P. and O.N. measured and analysed NMR and HR-MS data, respectively. J.B. wrote the manuscript with help of KD. All the authors read, edited and approved the final version of the manuscript.

Competing interests. We declare we have no competing interests.

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